The Effect of Thiourea on Ureide Metabolism in *Neurospora crassa*

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The wild-type strain of *Neurospora crassa* Em 5297a can utilize allantoin as a sole nitrogen source. The pathway of allantoin utilization is via its conversion into allantoic acid and urea, followed by the breakdown of urea to ammonia. This is shown by the inability of the urease-less mutant, *N. crassa* 1229, to grow on allantoin as a sole nitrogen source and by the formation of allantoate and urea by pre-formed mycelia of this mutant. In the wild strain (Em 5297a) thiourea is tenfold more toxic on an allantoin medium than on an inorganic nitrogen medium; allantoin as well as urea counteract thiourea toxicity in the allantoin nitrogen medium. This selective toxicity of thiourea for the mould utilizing allantoin nitrogen does not, however, result in an impairment of allantoin uptake, allantoinase activity or the formation of urea from allantoin. The only process affected by thiourea is the synthesis of urease; urea antagonizes this effect of thiourea in *N. crassa*.

Thiourea derivatives are toxic to plants and fungi (Barratt & Horsfall, 1947; Ludwig *et al.*, 1954; Rich & Horsfall, 1954; Ross & Ludwig, 1957) but the mechanism of their toxicity is virtually unknown. One of the few well-established effects of thiourea derivatives on nitrogen metabolism is the inhibition of soil nitrification by thiourea and allylthiourea. This has been ascribed to the toxicity of these compounds to soil *Nitrosomonas* and it has been suggested that complex-formation with copper ions may be involved (Lees, 1963).

During our investigations on the utilization of ureido compounds such as allantoin and urea by *Neurospora crassa*, we have observed a specific effect of thiourea on ureide metabolism. In this paper we show that thiourea is tenfold more toxic to *N. crassa* growing on allantoin as a sole nitrogen source than it is to the mould utilizing inorganic nitrogen compounds, and that this selective toxicity is directly due to an inhibition of urease synthesis. A preliminary report of some of these findings has appeared (Nirmala & Sivarama Sastry, 1972a).

Experimental

Chemicals

Solutions used for media were made up in glass-distilled water, and all chemicals were of analytical grade. Allantoin was a product of E. Merck A.G., Darmstadt, Germany, and thiourea was purchased from Sarabhia M. Chemicals Ltd., Baroda, India. These were autoclaved separately and added aseptically.

Glass powder used in enzyme extractions was prepared as follows. Pyrex glass was powdered in a ball mill and sieved to obtain a powder (50–100 mesh). The powder was left in conc. HNO_3 –HCl (1:1, v/v) for 18–20 h. It was then repeatedly washed with glass-distilled water till completely free of acid and dried in an oven at 100°C.

Methods

Organisms, media and growth. In most experiments, the wild-type strain *Neurospora crassa* Em 5297a was used. It was grown and maintained as described before (Sivarama Sastry *et al.*, 1962; Venkateswerlu & Sivarama Sastry, 1973). In some studies in which a urease-less mutant, *N. crassa* 1229, was used, identical procedures were employed as with the wild-type strain.

In growth experiments, the moulds were cultured in Pyrex conical flasks (50 ml) containing 10 ml of the culture medium (the 'normal magnesium' basal medium of Sivarama Sastry *et al.*, 1962) for 3 days at $30 \pm 1^\circ\text{C}$, at pH 4.8–5.0. The mycelia were harvested, washed thoroughly with water, dried at 60–80°C overnight and weighed to determine growth. Growth experiments were repeated a minimum of four times. In experiments wherein ureides were employed as the sole nitrogen sources, the ammonium nitrate and ammonium tartrate of the basal medium were replaced by suitable concentrations of allantoin or urea, which were minimal and adequate for achieving optimum growth of the mould.

Allantoin uptake by mycelia. *N. crassa* Em 5297a was grown for 48 h with allantoin (5 mg/10 ml) as sole

nitrogen source with or without 500 μ g of thiourea/10 ml of medium. Mycelia were harvested, repeatedly washed with sterile ice-cold water, transferred aseptically to a nitrogen-free basal medium and allowed to 'starve' for 18 h at 30°C; mycelia from cultures grown with thiourea were 'starved' similarly, but in the presence of the same concentration of thiourea (500 μ g/10 ml of medium). Preliminary experiments showed that this time was adequate to exhaust endogenous growth substrates. The mycelia (approx. 400 mg fresh wt.) were then washed with sterile ice-cold water and suspended in 50 ml of nitrogen-free basal medium supplemented with 500 μ g of allantoin/ml at 30 $\pm$ 1°C, and incubated with shaking on a mechanical shaker at 10–15 strokes/min with a horizontal stroke distance of 10 cm. Portions were removed at intervals and allantoin remaining in the medium was determined according to the 'direct' procedure of Nirmala & Sivarama Sastry (1972b).

In experiments wherein allantoin uptake and its conversion into allantoate and urea were studied in the urease-less mutant, *N. crassa* 1229, similar procedures were employed except that the mutant was first grown on the usual basal medium (Sivarama Sastry *et al.*, 1962) with or without 5 mg of thiourea/10 ml of medium for 48 h. Allantoin uptake and allantoate formation were determined by analysing the medium by the 'direct' procedure of Nirmala & Sivarama Sastry (1972b). Urea formed was estimated with the aid of jackbean urease. For this purpose, 1 g of jack-bean meal (Difco Laboratories, Mich., U.S.A.) was stirred with 100 ml of 70% (v/v) glycerol for 1 h at 30°C. The extract was centrifuged (5000g; 15 min) and the supernatant was used as the source of urease. Portions of the medium, at the end of flotation, were incubated, as described below, with 1 ml of urease solution for 30 min and the amount of NH₃ formed was measured.

Determination of thiourea. Thiourea uptake by *N. crassa* was determined by measuring thiourea remaining in the medium at the end of the growth period. The following procedure, based on that of Hutchinson & Boltz (1958), was employed. A suitable portion of the medium (containing 50–400 μ g of thiourea) was made up to 4 ml and 0.5 ml of sodium acetate–acetic acid buffer (0.05 M with respect to sodium acetate, pH 4.0) and 0.5 ml of NaNO₂ (1.6%, w/v) were added, mixed and the tubes then heated at 70°C for 30 min. After cooling to room temperature (30 $\pm$ 1°C), 0.5 ml of conc. HCl and 0.5 ml of FeCl₃ solution (3.2% FeCl₃·6H₂O in 0.04 M-HCl) were added, mixed well and absorbance was measured against a similarly run blank (no thiourea) at 485 nm in a Coleman Junior Spectrophotometer.

Enzyme assays. *N. crassa* mycelia (72 h old; approx. 500 mg fresh wt.) were extracted, by grinding, with twice their weight of glass powder and 6 ml of 0.14 M-diethanolamine–HCl buffer, pH 7.0, contain-

ing 10 μ M-thioglycollate and 0.1 mM-EDTA (disodium salt) for assay for allantoinase; extraction was done similarly but with 5 ml of phosphate buffer (Na₂HPO₄–NaH₂PO₄, 0.1 M, pH 7.0) containing 10 μ M-cysteine and 0.1 mM-EDTA (disodium salt) for assay of urease activity. All operations were performed at 0°C. The extracts were centrifuged (10000g, 15 min) and enzyme activities were determined in the supernatants as described below. Protein concentrations in mycelial extracts were determined by the method of Lowry *et al.* (1951) as modified by Oyama & Eagle (1956) with bovine serum albumin (Cohn fraction V; Mann Research Laboratories, New York, N.Y., U.S.A.) as standard.

Allantoinase activity was measured by incubating a suitable portion of the mycelial extract with allantoin (10 μ mol), EDTA (0.2 μ mol), thioglycollate (0.02 μ mol) and diethanolamine–HCl buffer (280 μ mol) in a total volume of 2 ml, at 30°C for 15 min. The reaction was stopped by the addition of 0.5 ml of 50% (w/v) trichloroacetic acid. Allantoate formed was determined according to the 'direct procedure' of Nirmala & Sivarama Sastry (1972b). Enzyme activity is expressed as μ mol of allantoate formed/15 min per mg of protein.

Urease activity was determined by incubating a suitable portion of the mycelial extract with urea (6.66 μ mol), EDTA (disodium salt, 0.2 μ mol), cysteine (0.02 μ mol) and phosphate buffer (100 μ mol of Na₂HPO₄–NaH₂PO₄, pH 7.0) in a total volume of 4.0 ml at 30°C for 30 min. Saturated K₂CO₃ (10 ml) was then added and the NH₃ formed was quantitatively collected by passage into 0.05 M-H₂SO₄ (10 ml). After neutralization, NH₃ was estimated by nesslerization. Enzyme activity is expressed as μ mol of urea decomposed/30 min per mg of protein.

When the effect of thiourea on mycelial urease activity was to be studied, the incubation mixture also contained 1 mM-thiourea.

Results

Growth of N. crassa on different nitrogen sources

The ability of *N. crassa* to utilize allantoin as a sole nitrogen source has not been studied hitherto. Hence *N. crassa* Em 5297a was allowed to grow on inorganic nitrogen (NH₄⁺ and NO₃[–]), allantoin or urea at different concentrations and growth was examined as a function of the nitrogen concentration in the medium in each case. The results obtained (Fig. 1) indicate that identical growth (40–50 mg dry wt.) is obtained with 4.25 mg of inorganic nitrogen, 2.0 mg of allantoin nitrogen (i.e. 5.64 mg of allantoin) or with 1.5 mg of urea nitrogen (i.e. 3.22 mg of urea) respectively per 10 ml of medium. In view of the efficiency with which allantoin is

Fig. 1. Effect of nitrogen sources on growth of *N. crassa* Em 5297a

For experimental details see the text. ●, Inorganic nitrogen (NH_4^+ and NO_3^-); ○, allantoin; ▲, urea. Growth is expressed as a function of the amount of nitrogen provided by each growth substrate.

utilized without any apparent lag period as a nitrogen source by *N. crassa*, it is evident that this mould possesses the enzyme systems required to degrade allantoin.

Thiourea toxicity

Thiourea toxicity on different nitrogen sources. Thiourea toxicity was next studied on the normal basal medium (inorganic nitrogen supplied as NH_4NO_3 plus ammonium tartrate; Sivarama Sastry *et al.*, 1962) and on medium containing allantoin (5mg/10ml) as sole nitrogen source; these two media will be referred to as 'inorganic nitrogen' and 'allantoin nitrogen' media respectively. Growth, as a function of increasing thiourea concentration, on

these two media is shown in Fig. 2. At a thiourea concentration of $5000\mu\text{g}/10\text{ml}$ growth is inhibited by 50% on the 'inorganic nitrogen' medium; in contrast, the corresponding concentration is $500\mu\text{g}$ of thiourea/10ml on the 'allantoin nitrogen' medium. In subsequent studies designed to elucidate the mechanism involved in thiourea toxicity these concentrations of thiourea have been employed to obtain half-maximal growth.

Reversal of thiourea toxicity by allantoin and urea. In view of the tenfold greater toxicity of thiourea to *N. crassa* growing on 'allantoin nitrogen' medium it appeared likely that thiourea specifically inhibits one or more steps involved in allantoin utilization in this mould. Experiments were therefore conducted to examine whether excess of allantoin or urea could counteract thiourea toxicity on the two media. From Fig. 3 it is evident that 10mg of allantoin/10ml of medium fully counteracts the growth inhibition caused by thiourea ($500\mu\text{g}$ of thiourea/10ml) on the 'allantoin nitrogen' medium. In contrast, there is little reversal of thiourea toxicity by allantoin on the inorganic nitrogen medium. Counteraction of thiourea toxicity on the allantoin nitrogen medium by urea and its relationship to thiourea uptake is shown in Fig. 4. Complete reversal of growth inhibition is obtained with $1500\mu\text{g}$ of added urea/10ml of medium, that is at a urea:thiourea ratio of 3:1. Concomitantly,

Fig. 2. Thiourea toxicity on the inorganic nitrogen and allantoin nitrogen media in *N. crassa* Em 5297a

For experimental details see the text. ●, Inorganic nitrogen medium; ○, allantoin nitrogen medium. Arrows indicate half-maximal growth and the concentrations of thiourea involved.

Fig. 3. Effect of allantoin on thiourea toxicity in *N. crassa* Em 5297a

For experimental details see the text. Concentrations of thiourea used for inhibition were 500 μ g/10ml on allantoin nitrogen medium (●), and 5000 μ g/10ml on inorganic nitrogen medium (○). The arrow represents growth of controls (no thiourea).

Fig. 4. Effect of thiourea toxicity in *N. crassa* Em 5297a on allantoin nitrogen medium

For experimental details see the text. The concentration of thiourea used for inhibition was 500 μ g/10ml. ●, Growth; ▲, thiourea uptake. The arrow indicates growth of control (no thiourea).

thiourea uptake by the mould is decreased from 15 μ g to about 7.5 μ g of thiourea/mg dry wt. of mycelium. Reversal of thiourea toxicity by urea on the inorganic nitrogen medium is shown in Table 1. These data reveal several interesting contrasts with the pattern indicated by the results in Fig. 4. First, complete restoration of growth is not obtained unless a much higher concentration of urea is present (urea:thiourea ratio required is 6:1). Secondly, this counteraction is now paralleled by a considerably greater decrease in mycelial thiourea uptake. It is apparent therefore that urea reverses thiourea toxicity at physiological concentrations only on the allantoin nitrogen medium and that reversal on the inorganic nitrogen medium is a less-specific effect probably directly related to an antagonism between urea and thiourea at the uptake level.

Allantoin uptake by *N. crassa* Em 5297a mycelia in thiourea toxicity. The possible metabolic changes in thiourea toxicity in *N. crassa* on the allantoin nitrogen medium were examined from several points of view. First, the uptake of allantoin by preformed *N. crassa* mycelia was studied. For this purpose, mycelia (48h old) of *N. crassa* Em 5297a grown on the allantoin nitrogen medium with or without toxic concentrations of thiourea (500 μ g/10ml) were starved and their ability to take up allantoin from the allantoin nitrogen medium was studied as a function of time. The results obtained (Fig. 5) show conclusively that thiourea toxicity does not affect this process.

Uptake of allantoin and its conversion into allantoin and urea in thiourea toxicity in *N. crassa* 1229.

Table 1. Effect of urea on thiourea toxicity in *N. crassa* Em 5297a on inorganic N medium

For experimental details see the text. Amounts of compounds supplemented are per 10ml of culture medium. The growth period was 72h at 30°C.

Supplements (mg)		Dry wt. (mg)	Uptake of thiourea (μ g/mg dry wt.)
Thiourea	Urea (control)		
None	—	46.8	—
5.0	—	19.2	104.2
5.0	10.0	38.1	16.4
5.0	30.0	45.4	6.9

Since the urease-less strain, *N. crassa* 1229, is known to accumulate urea in the medium when allowed to metabolize adenine and related purine derivatives (Davis, 1970), it was thought that this mutant could be employed to examine whether allantoin and urea were formed from allantoin. Growth experiments showed that, as in the wild-type strain *N. crassa* Em 5297a, in this mutant also 5 mg of thiourea/10ml of medium produced 50% growth inhibition on the inorganic nitrogen medium. (Thiourea toxicity could not be studied on the allantoin N medium in view of the inability of the mutant to grow under these conditions.) This mould was grown for 48h with (5mg/10ml) and without thiourea, the mycelia were starved as described earlier and floated on the

Fig. 5. Allantoin uptake by *N. crassa* Em 5297a mycelia in thiourea toxicity

For experimental details see the text. Mycelia (48 h old) were starved and refloated on nitrogen-free basal medium containing 500 μg of allantoin/10ml. ○, Control mycelia; ●, mycelia grown with 500 μg of thiourea/10ml of allantoin nitrogen medium.

Table 2. Formation of allantoate and urea in thiourea toxicity in the urease-less mutant, *N. crassa* 1229

Mycelia (2 days old), grown with or without 5mg of thiourea/10ml of inorganic nitrogen medium, were starved and floated on the allantoin nitrogen medium for 4h. For experimental details see the text. Values for metabolites are expressed as mg/4h per 100mg fresh wt. of mycelia.

Conditions of growth	Allantoin uptake	Allantoate formed	Urea formed
Control	2.68	0.096	1.45
Toxic	2.72	0.086	1.49

allantoin nitrogen medium for 4h. Uptake of allantoin and the formation of allantoate and urea were determined by analysis of the medium at the end of the flotation period. The results (Table 2) show that mutant 1229 does form allantoate and urea from allantoin; moreover, neither the uptake of allantoin nor its conversion into these two metabolites is impaired in mycelia grown in the presence of toxic concentrations of thiourea.

It may also be pointed out that comparison of the data of Fig. 5 and those of Table 2 shows that allantoin uptake is, in fact, much faster in mutant 1229 than in the wild-type strain Em 5297a. Hence

Table 3. Amounts of allantoinase and urease activity in thiourea toxicity on allantoin nitrogen medium in *N. crassa* Em 5297a

For experimental details see the text. Amounts of metabolites supplemented are per 10ml of culture medium. The growth period was 72h at 30°C. Allantoinase activity is expressed as μmol of allantoate formed/15min per mg of protein; urease activity is expressed as μmol of urea decomposed/30min per mg of protein.

Supplements to medium (μg)	Growth (mg dry wt.)	Enzyme activity	
		Allantoinase	Urease
Control (none)	45.3	0.177	3.10
Thiourea (500)	24.2	0.155	1.51
Thiourea (500) + urea (1500)	43.5	0.170	3.81

the mutation leading to loss of urease activity does not affect transport of allantoin.

Enzymes involved in ureide metabolism in N. crassa in thiourea toxicity. In view of the results presented above, it was decided to examine the activity *in vivo* of some enzymes involved in allantoin utilization in *N. crassa*. From Table 2 it is evident that the pathway

which is the general mode of degradation of allantoin (Reinbothe & Mothes, 1962), also obtains in *N. crassa*. The activities *in vivo* of allantoinase and urease in thiourea toxicity in *N. crassa* Em 5297a are shown in Table 3. It is noteworthy that, whereas allantoinase activity remains largely unaltered, urease activity is about 50% lower in thiourea toxicity. Moreover, under conditions of urea reversal, urease activity is concomitantly restored to normal. This decrease in urease activity, taken along with the other data detailed above, proves that the primary metabolic defect in thiourea toxicity is the decrease in urease activity and that it is this parameter that is growth-limiting on the allantoin nitrogen medium.

Thiourea has also been reported to inhibit *in vitro* the ureases of *Azotobacter vinelandii* (Mehta *et al.*, 1967) and *Corynebacterium renale* (Lister, 1956) at concentrations around 1mM: the *Azotobacter vinelandii* enzyme is 50% inhibited by 1mM-thiourea. Incubation with cysteine prevents this inhibition of the enzyme from both organisms. Hence *N. crassa* mycelial extracts were incubated with 1mM-thiourea for periods up to 30min, and then assayed for urease activity. The enzyme activity was totally unaffected, indicating that the decrease in urease activity (Table 3) is not explicable on the basis of any intracellular association between *N. crassa* urease and thiourea.

This was further confirmed by the finding that when extracts from *N. crassa* cultures grown with thiourea were incubated with cysteine (up to 2mM), no increase in activity was observed even on prolonged incubation with cysteine.

Discussion

Allantoin metabolism has been studied in plants and in several micro-organisms (Reinbothe & Mothes, 1962; Vogels, 1966; Van der Drift *et al.*, 1970) and it is generally believed that the transformations shown in Scheme 1 are involved. The alternative pathway involving steps 4 and 5 has been found, more recently, to be operative in *Streptococcus allantoicus* and other micro-organisms (Vogels, 1966; Van der Drift *et al.*, 1970).

The present investigation has revealed, for the first time, that wild-type *N. crassa* can grow on allantoin as a sole nitrogen source. The formation of the two metabolites, allantoate and urea, as obligatory intermediates is indicated by their accumulation in the urease-less mutant, *N. crassa* 1229, utilizing allantoin (Table 2) and the inability of this mutant to grow on the allantoin nitrogen medium. Although conversion of allantoate into urea is evident, the nature of the enzyme system(s) involved in this process is not clear, since we have been unable to obtain appropriate enzyme activity in mycelial crude extracts. In this context, it may be mentioned that such enzymes have occasionally been difficult to demonstrate. For instance, in *Fusarium moniliforme* (Allam & Elzainy, 1971) allantoicase activity is very weak and is assayable only in the presence of phenylhydrazine as a trapping agent. Allantoate amidohydrolase shows complex properties and needs a preactivation at a low pH quite removed from its region of activity (Vogels, 1966). It is quite likely that right conditions have not yet been found to demonstrate the allantoate-degrading enzymes in *N. crassa*.

An unusual and specific effect of thiourea on ure-

ide metabolism has been demonstrated, for the first time herein, in *N. crassa*. Thiourea is selectively more toxic to this mould on the allantoin nitrogen medium than on the inorganic nitrogen medium. This toxicity is unrelated to complex-formation with copper ions (see, for instance, Lees, 1963), since toxicity is decreased tenfold on the inorganic nitrogen medium, which differs from the allantoin nitrogen medium only in the nitrogen source. Thiourea specifically inhibits allantoin utilization in *N. crassa* by bringing about decreased activity of urease *in vivo*. The evidence in favour of this hypothesis consists, first, in the counteraction, by supplemented allantoin or urea, of the growth inhibition caused by thiourea. As would be expected, urea is more effective in this regard than allantoin; complete reversal is obtained with about 1.5mg of urea and with about 10mg of allantoin/10ml of medium. Secondly, in thiourea toxicity, allantoin uptake (Fig. 5), allantoicase activity (Table 3) and the formation of allantoate and urea (Table 2) all remain unaffected, the only parameter altered *in vivo* being the amount of urease activity.

The results presented here also indicate the mechanism involved in the observed changes in urease in the mould. Unlike the ureases of *A. vinelandii* (Mehta *et al.*, 1967) and *Corynebacterium renale* (Lister, 1956), *N. crassa* urease is not, apparently, inhibited by thiourea, since urease activity of mycelial extracts was totally unaffected by 1mM-thiourea. Moreover, incubation of mycelial extracts derived from thiourea-grown cultures, with cysteine (up to 2mM) does not enhance urease activity. Since when thiourea toxicity is overcome by urea supplement there is a concomitant restoration of urease activity to normal levels (Table 3), it is probable that the process affected is the synthesis of urease. In *A. vinelandii* (Mehta *et al.*, 1967) thiourea is an inducer of urease. With *N. crassa* thiourea functions in an opposite manner and probably inhibits urease synthesis.

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Scheme 1. Metabolism of allantoin

Enzymes involved: 1, allantoinase; 2 and 3, allantoicase; 4 and 5, allantoate amidohydrolase.

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