Degradation of Xanthine by *Penicillium chrysogenum*

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SUMMARY

Penicillium chrysogenum utilized the purines hypoxanthine, xanthine, uric acid and adenine as sole nitrogen sources but not the methylated purines caffeine and theobromine. Cell-free extracts of this organism contained the enzymes xanthine dehydrogenase, uricase, allantoinase, allantoicase and urease. Uric acid was degraded to allantoic acid by way of allantoin; allantoin was degraded to glyoxylic acid by way of allantoic acid. Xanthine dehydrogenase, uricase, allantoinase and urease were constitutive whereas allantoicase was inducible by xanthine or allantoin.

INTRODUCTION

Fungi utilize purines as a nitrogen source (DiCarlo, Schultz & McManus, 1951; Franke, Taha & Krieg, 1952; Wolf, 1955; Taha, Krieg & Franke, 1955; Taha & Sharabash, 1956; Sukhenko & Podgainaya, 1958; Kim & Wolf, 1961; Fukumoto, Watanabe & Yano, 1968; LaRue & Spencer, 1968). The utilization of these compounds presumably requires enzymes that catalyse the degradation of the purine skeleton to simpler compounds. Taha *et al.* (1955) reported xanthine oxidase in extracts of some fungal species. The enzyme uricase, which catalyses the degradation of uric acid to allantoin, has been shown to be present in many fungi (Brunel, 1937; Franke *et al.* 1952; Roush & Domnas, 1956; Laboureur & Langlois, 1967). The enzyme allantoinase, which catalyses the hydrolysis of allantoin to allantoic acid, was demonstrated in basidiomycetes (Brunel, 1937), *Aspergillus niger* (Brunel, 1931; 1939), *Candida utilis* and *Saccharomyces cerevisiae* (Lee & Roush, 1964). Allantoicase, catalysing the conversion of allantoic acid to glyoxylic acid and urea, was shown in *A. niger* (Brunel, 1939), *C. utilis* and *S. cerevisiae* (Domnas, 1962; Lee & Roush, 1964) and in *Penicillium notatum* and *P. citreo-viride* (Trijbels & Vogels, 1966). These enzymic studies do not give a complete picture of the degradation of purines in one single organism. The present work aims to elucidate the enzymic steps in the degradation of xanthine by extracts of *P. chrysogenum*.

METHODS

Organism. *Penicillium chrysogenum* was obtained from the centre of cultures of the National Research Centre of Egypt.

Media. The organism was cultivated and kept on slants of solid Czapek-Dox medium. This medium contained (% w/v): glucose, 3; K_2HPO_4 , 0.1; $MgSO_4 \cdot 7H_2O$, 0.05; KCl, 0.05; $NaNO_3$, 0.2; agar 2.0. The liquid medium used for growing the

organism was the same with the appropriate purine replacing NaNO_3 on an equivalent nitrogen basis. Purines and related compounds were sterilized by filtration but all other ingredients of the medium were autoclaved.

Culture and harvest of mycelium. Conidia were scraped from mycelium which had been grown on slopes for 7 days at 28° and suspended in cold sterile distilled water. One ml. samples of this suspension were used to inoculate 250 ml. Erlenmeyer flasks each containing 50 ml. sterile medium. The inoculated flasks were incubated at 28° for 3 to 4 days, then the mycelium was harvested by filtration, washed thoroughly with distilled water and finally blotted dry with absorbent paper.

Preparation of cell-free extracts. The blotted-dry mycelium was ground with about twice its weight of washed cold sand and extracted with 0.05 M potassium phosphate buffer (pH 7.4). The slurry was centrifuged at 8,000 rev./min. for 20 min. and the supernatant liquid used as the crude enzyme preparation. During this operation the containers and solutions were kept at 0° .

Preparation of potassium allantoate. This was prepared by the alkaline hydrolysis of allantoin (Young & Conway, 1942).

Enzyme assays. Xanthine dehydrogenase was assayed either by estimating the uric acid formed during the incubation of cell-free extracts with xanthine (Blauch & Koch, 1939), or by the anaerobic reduction of 2,3,5-triphenyltetrazolium chloride by the Thunberg tube technique and estimating the formazan formed (Kun & Abood, 1949). Uricase was assayed by measuring the disappearance of uric acid and the formation of allantoin during the incubation of extracts + uric acid; the allantoin formed was estimated by the method used by Franke *et al.* (1952). Allantoinase activity was measured by following the formation of allantoic acid when allantoin was incubated with extracts. Allantoic acid was estimated by the same procedure used for allantoin with the omission of alkaline hydrolysis. Allantoicase was assayed by following glyoxylic acid formation when potassium allantoate was incubated with the extracts. Glyoxylic acid was estimated by the procedure used for allantoin with the omission of alkaline and acid hydrolysis. Urease activity was determined by estimating the ammonia formed from urea using Nessler's reagent.

Protein determination. This was done by the method of Sutherland, Cori, Haynes & Olsen (1949).

Identification of products. Allantoin and allantoic acid were recognized by ascending paper chromatography on Whatman No. 1 paper and two different solvent systems: *n*-butanol + ethanol + water (4 + 1 + 1 by vol.) with R_f values of 0.13 and 0, respectively (the yellow spot of sprayed allantoic acid was located on the start line); phenol + 10 % sodium citrate (5:1, w/v), R_f values 0.41 and 0.17 (Sukhenko & Podgainaya, 1958). After drying the chromatograms at room temperature they were sprayed with Ehrlich's reagent (Smith, 1960); identifications were checked by co-chromatography with authentic standards. Glyoxylic acid was converted to the 2,4-dinitrophenylhydrazone (Smith & Smith, 1960), chromatographed on Whatman No. 1 paper with two different ascending solvent systems: *n*-butanol + ethanol + water (70 + 10 + 20 by vol.) where the R_f was 0.35 and *n*-butanol + ethanol + 0.5 N- NH_4OH (70 + 10 + 20 by vol.), R_f = 0.39 (Smith & Smith, 1960). The hydrazone of the authentic acid was prepared and run as control.

RESULTS

Growth of Penicillium chrysogenum on different purines and other nitrogenous compounds. Table 1 demonstrates that allantoin yielded the best growth followed by hypoxanthine and adenine. Xanthine and uric acid supported growth which was more or less equal to that obtained on sodium nitrate. The methylated purines caffeine and theobromine were not utilized. Kim & Wolf (1961) found that methylated purines were not utilized by *P. chrysogenum* Q-176. The feeble growth obtained when ammonium chloride was sole nitrogen source might be attributed to the decrease of pH value of the culture medium due to utilization of the ammonium ions.

Table 1. *Growth of Penicillium chrysogenum on different purines and other nitrogenous compounds*

Triplicate culture flasks contained 50 ml. of Czapek-Dox medium in which NaNO_3 was replaced by a nitrogen-equivalent amount of each nitrogen source, incubated 8 days at 28°.

Nitrogen source	Mean mycelium dry weight (mg./flask)
Adenine	338.9
Hypoxanthine	345.0
Xanthine	249.9
Uric acid	274.5
Caffeine	0
Theobromine	0
Allantoin	392.3
Urea	220.0
NaNO_3	276.7
NH_4Cl	187.7

Table 2. *Xanthine dehydrogenase in extracts of Penicillium chrysogenum mycelium*

Duplicate Thunberg tubes under vacuum contained xanthine (where indicated), 45 to 50 μmoles ; 2,3,5-triphenyltetrazolium chloride, 1.0 μmole ; sodium pyrophosphate buffer, pH 8.6, 140 μmoles ; extract, equiv. 3 to 4.4 mg. protein; total volume, 3.1 ml. (Expt. 1), 4.0 ml. (Expt. 2); temp. 37°; reaction time, 120 min.

Expt. no.	Additions	$\mu\text{g. Formazan formed}$
1	Xanthine-grown mycelial extract { Xanthine	145.3 ± 1.4
	{ —	53.6
2	Nitrate-grown mycelial extract { Xanthine	122.1 ± 3.3
	{ —	72.6 ± 6.6

Xanthine dehydrogenase. Table 2 shows xanthine dehydrogenase activity in extracts of *Penicillium chrysogenum* grown with xanthine or sodium nitrate. In the absence of xanthine the formazan formed ranged from 36 to 59 % of that produced when xanthine was present. Xanthine dehydrogenase was constitutive since it was present when the organism was grown with nitrate as sole nitrogen source. The data in Table 2 indicate that enzymic dehydrogenation of xanthine by extracts of the experimental organism is a slow process. This could be due to the partial loss of a certain requirement of the enzyme in the preparation of the extract. This point was tested by observing the formation of uric acid from xanthine: in comparable conditions to

those in Table 2 (but only 20 μ moles xanthine), only 0.25 μ mole of uric acid was detected after 1.5 hr and 0.45 μ mole after 3 hr. No significant uric acid was detected at the beginning of the reaction.

Uricase and combined uricase-allantoinase activities. Figure 1 shows the disappearance of uric acid catalysed by extracts of mycelia grown with xanthine or with nitrate; this shows that 7.9 μ moles of uric acid had almost disappeared in 150 min. with xanthine-grown mycelium. Analysis of this reaction mixture showed 7.12 μ moles

Fig. 1

Fig. 2

Fig. 1. Disappearance of uric acid catalysed by extracts of *Penicillium chrysogenum* grown with xanthine (○) or nitrate (●). Reaction mixture contained uric acid, 7.9 to 12.0 μ moles; sodium pyrophosphate buffer, pH 8.6, 60 μ moles; extract, 9 to 10 mg. protein; total volume, 4 ml.; temp. 37°.

Fig. 2. Allantoinase in extracts of *Penicillium chrysogenum* grown with xanthine (○), allantoin (Δ) or nitrate (●). Reaction mixture contained allantoin, 2 to 2.5 μ moles/ml.; extract, equiv. 0.85 mg. protein/ml. (○), 1.1 mg./ml. (Δ), 2.25 mg./ml. (●); volume 4 to 8 ml.; temp. 37°.

allantoin, 1.05 μ moles allantoate, no uric acid or glyoxylate. The formation of allantoic acid indicated the presence of allantoinase which hydrolysed part of the allantoin formed to allantoic acid. Hence allantoin is an intermediate in the degradation of uric acid to allantoic acid. Glyoxylic acid was not detected; it seems that this acid was formed only when higher concentrations of allantoic acid were present. Figure 1 indicates that uricase is a constitutive enzyme in *Penicillium chrysogenum* since extracts prepared from mycelia grown on nitrate were active. Franke *et al.* (1952) showed that uricase is a constitutive enzyme in *Aspergillus niger* and *Alternaria porri*. On the other hand, Roush & Domnas (1956) reported that uricase is an inducible enzyme in *Torulopsis utilis* and Fukumoto *et al.* (1968) demonstrated that uricase was induced by uric acid in a *Streptomyces* species.

Allantoinase. Figure 2 shows the hydrolysis of allantoin to allantoic acid by extracts of mycelia grown with xanthine, with allantoin or with nitrate. The different rates were probably due to variations of the amounts of extract protein present in the

reaction mixture. These results indicate that the enzyme is constitutive in *Penicillium chrysogenum*. Lee & Roush (1964) reported that allantoinase was induced by uric acid, allantoin or allantoic acid in *Candida utilis* whereas this enzyme was constitutive in *Saccharomyces cerevisiae*.

Allantoicase. Figure 3 demonstrates the presence of this enzyme in extracts of xanthine-grown and allantoin-grown *Penicillium chrysogenum*. Urea, which is the other expected product of this reaction, was not determined because of a very active urease (see below).

Fig. 3

Fig. 4

Fig. 3. Allantoicase in extracts of *Penicillium chrysogenum* grown with xanthine (○) or allantoin (●). Reaction mixture contained potassium allantoate 32 μmoles; potassium phosphate buffer, pH 7.4, 100 μmoles; extract, equiv. 7.2 to 9.0 mg. protein; total volume, 3 ml.; temp. 37°.

Fig. 4. Combined allantoinase and allantoicase activities in extracts of *Penicillium chrysogenum* grown with xanthine. Reaction mixture contained allantoin, 200 μmoles; potassium phosphate buffer, pH 7.0, 100 μmoles; extract, equiv. 7.2 mg. protein; total volume, 5 ml.; temp. 37°. ●, Allantoate formed; ○, glyoxylate formed.

Trijbels & Vogels (1966) demonstrated allantoicase activity in extracts of *Penicillium notatum* and *P. citreo-viride* grown in an allantoin + yeast extract medium. The two penicillia were reported to be urease negative. These authors reported that ureidoglycollic acid is an intermediate in the degradation of allantoic acid to glyoxylic acid and urea. We tried to detect this intermediate in the reaction by their procedure making use of a neutral hydrolysis of ureidoglycollic acid to glyoxylic acid but unreproducible results were obtained.

Growing the experimental organism on nitrate yielded extracts lacking allantoicase activity. This shows that allantoicase is an inducible enzyme. Lee & Roush (1964) showed that, while allantoicase was induced by uric acid, allantoin or allantoic acid in *Candida utilis*, the same enzyme was constitutive in *Saccharomyces cerevisiae*.

Combined allantoinase and allantoicase activities. In this experiment allantoin was incubated with extracts of mycelia grown with xanthine as sole nitrogen source and

both allantoic and glyoxylic acids were determined. As shown in Fig. 4, glyoxylic acid started to appear in significant amounts after the formation of substrate amounts of allantoic acid. This experiment shows that allantoic acid is an intermediate in the degradation of allantoin to glyoxylic acid.

Urease. Figure 5 shows urease activity in extracts of this organism grown with xanthine. The enzyme is very active since about 90 μ moles of ammonia were formed in 12 min. Comparable urease activity was found in extracts of mycelia grown with allantoin or nitrate. Hence the enzyme is constitutive. The enzyme tolerated high concentrations of urea; in one experiment 300 μ moles of urea were added initially and fairly good enzymic activity was obtained.

Fig. 5. Urease in extracts of *Penicillium chrysogenum* grown with xanthine. Reaction mixture contained urea, 60 μ moles; potassium phosphate buffer, pH 7.0, 100 μ moles; extract, equiv. 7.2 mg. protein; total volume, 3 ml.; temp. 37°.

DISCUSSION

Penicillium chrysogenum 65 grown on Czapek-Dox medium with xanthine as sole nitrogen source degraded xanthine by a multiple enzyme system as follows:

From uric acid onwards, this degradation sequence is similar to that demonstrated in aerobic bacteria (Franke & Hahn, 1955; Campbell, 1955). The fate of glyoxylic acid, which is the other product of this degradation, needs further investigation; it could be further oxidized to oxalic acid by a system similar to that reported by Franke & Hahn (1955) in *Pseudomonas aeruginosa* or to formic acid and CO₂ as demonstrated by Campbell (1955), with extracts of an unidentified *Pseudomonas* sp. However, the possibility exists that this two-carbon compound could also be utilized in the synthesis of cellular compounds, e.g. the formation of glycine by the reductive amination of glyoxylic acid. It might also be utilized through the glyoxylate cycle if it is operating in this organism.

Xanthine dehydrogenase, uricase and allantoinase were constitutive enzymes but allantoinase seems to be inducible, being induced by xanthine or allantoin. The constitutive enzymes are presumably important to the organism; their combined activities may play a central role in the control of the concentration of free purines in the cell pool. On the other hand, when the organism is grown on the purine as its sole nitrogen source, all the enzymes, including allantoinase, are required to catalyse the complete degradation of the purine to glyoxylic acid and ammonia.

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