The degradation of paracetamol (4-hydroxyacetanilide) and other substituted acetanilides by a *Penicillium* species

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A mould which was isolated from a solution of paracetamol was identified as a *Penicillium* species and was found to possess the ability to utilise a series of substituted acetanilides, including paracetamol (4-hydroxyacetanilide), phenacetin (4-ethoxyacetanilide) and metacetamol (3-hydroxyacetanilide) as sole carbon sources for growth. Studies with washed-cell suspensions indicated that growth of the *Penicillium* isolate in the presence of paracetamol induced the respective enzyme systems for the degradation of this compound. Manometric studies, measuring oxygen uptake rates, indicated that the mould was capable of degrading paracetamol to acetate and 4-aminophenol. Acetate was further metabolised whilst 4-aminophenol accumulated in the growth medium and was subsequently identified by UV spectroscopy and thin-layer chromatography. Similar experiments with phenacetin indicated metabolism by the mould to acetate and 4-ethoxyaniline which was isolated and identified by subsequent analysis of the growth medium. However, unlike 4-aminophenol and 4-ethoxyaniline, the degradation product (3-aminophenol) from metacetamol metabolism was further degraded by the mould.

INTRODUCTION

A variety of microorganisms, including bacteria and fungi, have been shown to metabolise and utilise as sole sources of carbon or nitrogen both aliphatic and aromatic amides. Sharabi and Bordeleau (1969) isolated from soil, species of *Penicillium* and *Pullularia* which were able to degrade the herbicide N-(3,4-dichlorophenyl)-2-methylpentanamide (Karsil) and related compounds. Similar degradations of acylanilide herbicides by microorganisms have been reviewed by Bartha and Pramer (1970).

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The utilisation of amides as nitrogen sources by *Aspergillus nidulans* has been studied by Hynes and Pateman (1970) whilst Betz and Clarke (1973) showed that strains of *Pseudomonas putida*, *Pseudomonas cepacia* and *Pseudomonas acidivorans* could grow in a mineral salts medium with acetamide or phenylacetamide as the carbon or nitrogen source. A strain of *Corynebacterium pseudodiphtheriticum* was shown to possess the ability to degrade a number of N-arylacylamides of pharmaceutical, veterinary and agricultural interest (Grant and Wilson, 1973).

This report describes the ability of a *Penicillium* species to degrade a series of substituted acetanilides including paracetamol and phenacetin.

MATERIALS AND METHODS

Chemicals. Chemicals used in the experimental methods were obtained from Aldrich Chemical Co. Ltd., Milwaukee, U.S.A.; Ralph N. Emanuel Ltd., Wembley, Middlesex; Koch Light Laboratories Ltd., Colnbrook, Bucks.; May and Baker Laboratory Chemicals, Dagenham; and Phase Separations, Queensferry, Flints.

4-Hydroxy, N-methylacetanilide was prepared using the method of Julia and Bagot (1964).

Microbiological media were obtained from Oxoid Ltd., London

Growth media. A basal mineral salts medium having the following composition was used: $NaNO_3$, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; KH_2PO_4, 3.5 g; Na_2HPO_4, 0.5 g; distilled water to 1 litre. The medium was sterilised at 121 C for 20 minutes.

For the carbon utilisation experiments the medium was supplemented with the appropriate carbon compound at a concentration of 0.01% ($^w/_v$). Solutions of the carbon compounds were sterilised separately by membrane filtration before adding aseptically to the basal mineral salts medium. The pH of the final medium was 5.8–6.0.

Isolation and maintenance of the mould. The mould was isolated from an acidic solution of paracetamol (4-hydroxyacetanilide) which had been stored in the laboratory at 25 C for 3–5 days. Growth was established on malt agar (Oxoid) after incubation of the mould at 25 C. Subsequent identification showed the mould to be a *Penicillium* species. Stock cultures were maintained on malt agar at 4 C after initial growth at 25 C until required for use.

Preparation of washed-cell suspensions.

a. for carbon utilisation experiments. The *Penicillium* isolate was grown in mineral salts medium, containing acetamide 1% ($^w/_v$) as carbon source, at

25 C for 14 days. After incubation the cells were collected by centrifugation and washed three times using quarter-strength Ringer's solution. The cells were resuspended in quarter-strength Ringer's solution and the turbidity of this suspension adjusted such that on addition of one drop (0.05 ml) of the suspension to 20 ml of mineral salts medium, no visible turbidity was produced.

b. for manometric experiments. Cells not adapted to paracetamol metabolism were grown at 25 C for 7 days in mineral salts medium containing acetamide 1 % (w/v) as carbon source. Triple-washed-cell suspensions were prepared in quarter-strength Ringer's solution and the cell concentration adjusted to 10 mg dry weight cells/ml.

Cells adapted to metabolise paracetamol were similarly prepared after growth at 25 C in mineral salts medium containing both paracetamol 0.1 % (w/v) and acetamide 1 % (w/v) as carbon sources.

Growth with various carbon sources. 20-ml aliquots of mineral salts medium containing the appropriate compound 0.01 % (w/v) as carbon source were inoculated with one drop (0.05 ml) of the washed-cell suspension and incubated at 25 C for 14–21 days. Growth of the *Penicillium* species was estimated visually. 20-ml aliquots of mineral salts medium containing no added carbon source and 20-ml aliquots of mineral salts medium containing both added carbon source 0.01 % (w/v) and acetamide 1 % (w/v) were simultaneously inoculated and incubated to check for non-carry over of nutrients from the washed-cell suspension and absence of toxicity of the added carbon source respectively. Uninoculated controls were similarly prepared.

400-ml aliquots of mineral salts medium containing paracetamol 0.01 % (w/v) and 0.1 % (w/v) or phenacetin 0.01 % (w/v) and 0.1 % (w/v) as sole carbon sources were similarly prepared, inoculated and incubated at 25 C in baffled Erlenmeyer flasks (1 litre) in an orbital incubator (Gallenkamp Ltd., London) at a shaking speed of 120 rpm. Growth was estimated visually during the incubation period (14–21 days) and the concentration of paracetamol and phenacetin monitored every 3 days by sampling aliquots of the medium, removing the cells by centrifugation and analysing the supernatant by UV spectroscopy at the appropriate wavelength. Inoculated and uninoculated control solutions were similarly prepared and analysed.

Manometric techniques. Conventional manometric techniques similar to those of Umbreit, Burris and Stauffer (1972) were used. Constant-volume respirometers and single-side-arm flasks were used. Each flask contained 1.0 ml of 0.1 M phosphate buffer (pH 5.8) and 1.0 ml of an aqueous solution of the substrate (1–3 μmole) in the main compartment, 0.5 ml cell suspension containing 8–10 mg dry-weight cells/ml in the side arm and 0.2 ml KOH 20 % (w/v) in the centre well. The gas phase was air and the bath temperature 25 C.

Chemical determinations.

Thin-layer chromatography (TLC). Solutions of paracetamol 0.1% (w/v) and phenacetin 0.1% (w/v) which had been inoculated and incubated with the *Penicillium* species were analysed by TLC for the presence of possible degradation products.

After removal of the cells by centrifugation the supernatant was divided into two parts. One part was adjusted to pH 2.0 with HCl (5N) and the other to pH 11.0 with NaOH (5N). Each solution was extracted with ethyl acetate. 50 µl of the ethyl acetate extract was spotted onto silica-gel plates which had been activated at 100 C for 45 minutes. The chromatograms were developed using a method similar to that of Smith and Rosazza (1974) and one of the following solvent systems: benzene–methanol (95:5), benzene–methanol–acetic acid (45:8:4) or toluene–piperidine (5:2). The chromatograms were sprayed with ferric nitrate solution 5% (w/v) and dried at 45 C for 15 min to develop the spots. Possible degradation products were compared with authentic samples treated in a similar manner.

Isolation and identification of 4-ethoxyaniline from phenacetin degradation. Isolation of 4-ethoxyaniline from phenacetin degradation was performed using a method similar to that of Grant and Wilson (1973). Cultures (800 ml) grown in the presence of phenacetin 0.1% (w/v) for 14 days in an orbital incubator at 25 C were centrifuged and the cells removed. The supernatant liquid was extracted once with ether (160 ml) and the ethereal extract dried over anhydrous sodium sulphate. The ether was distilled off at 25 C under reduced pressure leaving a brown liquid residue. HCl (2N) was added dropwise to produce a crystalline hydrochloride. Impurities were removed by shaking with chloroform (10 ml) four times. The crystals were dissolved in hot ethanol, decolorised with charcoal and recrystallised. The authenticity of the crystallised product was checked by UV and IR analysis, melting point and TLC and compared with a known sample of 4-ethoxyaniline treated in the same way.

RESULTS

Growth with various compounds as sole carbon source. Table 1 indicates the ability of the *Penicillium* isolate to utilise a variety of aliphatic and aromatic compounds, in particular the substituted acetanilides including paracetamol, phenacetin and metacetamol, as sole carbon sources for growth at concentrations of 0.01% (w/v). Of the compounds tested only 2-aminophenol, 4-aminophenol, 4-ethoxyaniline and nicotinic acid did not support the growth of the mould at concentrations of 0.01% (w/v). Absence of toxicity of

Table 1. Compounds utilised by the *Penicillium* isolate as sole carbon sources for growth

Acetanilide	
Substituted acetanilides	*Amides*
4-Hydroxyacetanilide (paracetamol)	Acetamide
4-Ethoxyacetanilide (phenacetin)	Benzamide
3- Hydroxyacetanilide (metacetamol)	Nicotinamide
	Salicylamide
4-Chloroacetanilide	Propionamide
4-Iodoacetanilide	*n*-Butyramide
4-Bromoacetanilide	
4-Nitroacetanilide	*Other compounds*
4-Methylacetanilide	Formate
4-Aminoacetanilide	Acetate
4-Formylacetanilide	*n*-Propionate
4-Methoxyacetanilide	*n*-Butyrate
4-Carboxyacetanilide	
2-Carboxyacetanilide	Phenylacetate
2-Ethoxyacetanilide	Glycine
2-Hydroxyacetanilide	*n*-Acetylglycine
N-Methylacetanilide	3-Aminophenol
4-Hydroxy, N-methylacetanilide	

nicotinic acid at this concentration towards the *Penicillium* isolate was indicated by the ability of the mould to grow in the presence of nicotinic acid 0.01% ($^{w}/_{v}$) and acetamide 1% ($^{w}/_{v}$) when both these compounds were included as carbon sources. However, a reduced amount of growth was observed when 2-aminophenol, 4-aminophenol or 4-ethoxyaniline at a concentration of 0.01% ($^{w}/_{v}$), was included with acetamide 1% ($^{w}/_{v}$) in the growth medium, compared with the amount of growth obtained when acetamide 1% ($^{w}/_{v}$) was present as the sole carbon source for growth. Thus, these three compounds have some apparent toxic effect towards the mould.

UV analysis of a solution of paracetamol which had been inoculated with the *Penicillium* species showed a marked decrease in the concentration of paracetamol during the incubation period of 14–21 days and a characteristic shift in spectrum from the spectrum which had been initially recorded for paracetamol (Fig. 1A). Similar changes in spectrum were obtained for phenacetin solutions (Fig. 1B). In addition to this change in spectrum the inoculated solutions of paracetamol and phenacetin darkened appreciably during the incubation period. Uninoculated control solutions showed no apparent loss of of paracetamol or phenacetin from solution and no darkening of these solutions was observed during the same period of incubation.

Utilisation of some substituted acetanilides and related compounds by washed cell suspensions. Table 2 shows that *Penicillium* cells grown with acetamide

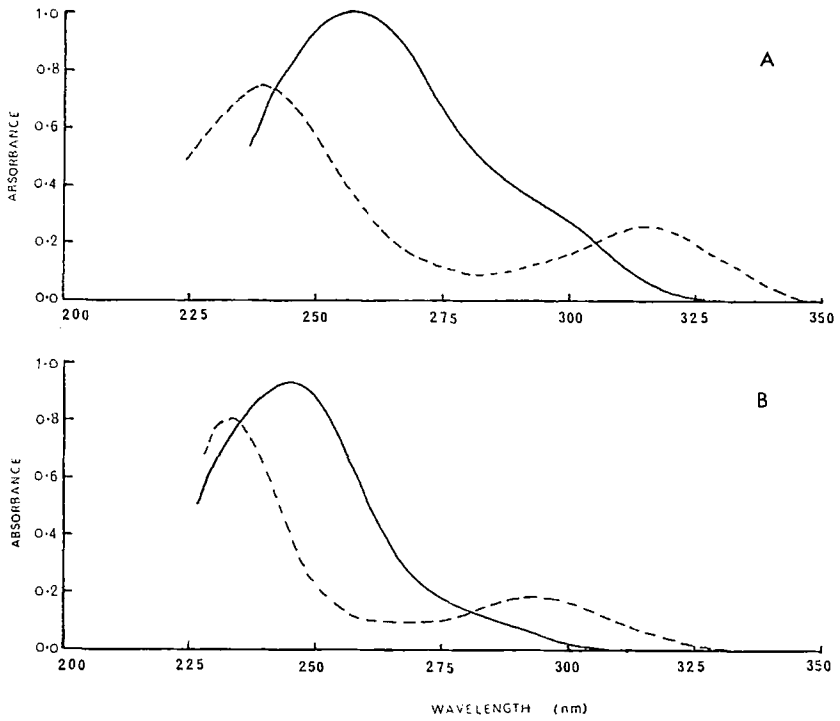


Fig. 1A. Spectral changes which occurred during growth of the *Penicillium* isolate with 4-hydroxyacetanilide (paracetamol). ——— paracetamol, - - - - - degradation product.
B. Spectral changes which occurred during growth of the *Penicillium* isolate with 4-ethoxyacetanilide (phenacetin). ——— phenacetin, - - - - - degradation product.

oxidised 3-aminophenol, 4-ethoxyacetanilide (phenacetin), 3-hydroxyacetanilide (metacetamol) and 4-hydroxyacetanilide (paracetamol) only after definite lag periods whilst acetamide and acetate were both oxidised immediately. However, cell suspensions which had been grown in the presence of both acetamide and paracetamol oxidised 3-aminophenol, 4-ethoxyacetanilide, 3-hydroxyacetanilide and 4-hydroxyacetanilide as well as acetate and acetamide without the presence of an initial lag period, indicating that induction of enzyme systems for the metabolism of the compounds had occurred during prior incubation of the cells with paracetamol (Table 2). The substrates 4-aminophenol and 4-ethoxyaniline were not metabolised either by acetamide- or paracetamol-acetamide-grown cells. Final oxygen uptakes for paracetamol (1 μmole) metabolism by acetamide-grown cells were similar to those obtained for the metabolism of acetate (1 μmole) and acetamide (1 μmole) indicating the oxida-

Table 2. The oxidation of some acetanilides and related compounds by washed cell suspensions of *Penicillium* species

Substrate	Cells grown in the presence of			
	acetamide 1% (w/v)		acetamide 1% (w/v) + paracetamol 0.1% (w/v)	
	Lag period (min)	O_2 uptake[1] (μmole/μmole substrate)	O_2 uptake[1] (μmole/μmole substrate)	
Acetate	No lag	1.1	1.2	
Acetamide	No lag	1.1	1.2	
3-Aminophenol	10	1.4	1.4	
4-Aminophenol	–	NM	NM	
4-Ethoxyaniline	–	NM	NM	
4-Ethoxyacetanilide (phenacetin)	10	2.2	1.9	
3-Hydroxyacetanilide (metacetamol)	12	2.6	2.6	
4-Hydroxyacetanilide (paracetamol)	12	1.2	0.8	

[1] Endogenous oxygen uptake subtracted.
NM: not metabolised.

tion of the acetyl moiety of the paracetamol molecule. Similar final oxygen uptakes were also obtained for the metabolism of acetate (1 μmole) and acetamide (1 μmole) by paracetamol-acetamide-grown cells. Paracetamol (1 μmole) oxidation by the same paracetamol-adapted cells was suppressed and this may be due to repression by the degradation products of paracetamol metabolism, since 4-aminophenol showed some toxicity towards the *Penicillium* species during growth. Final oxygen uptakes for metacetamol (1 μmole) oxidation were higher than those indicated for acetate (1 μmole) or acetamide (1 μmole) metabolism, since the degradation product 3-aminophenol was also shown to be oxidized by the *Penicillium* cells, thus accounting for this increased oxygen uptake with both non-adapted and paracetamol-adapted cells. No explanation can be given at the moment for the increased oxygen uptake obtained for the oxidation of phenacetin (1 μmole) since the degradation product, 4-ethoxyaniline, was not apparently oxidised by the *Penicillium* cells.

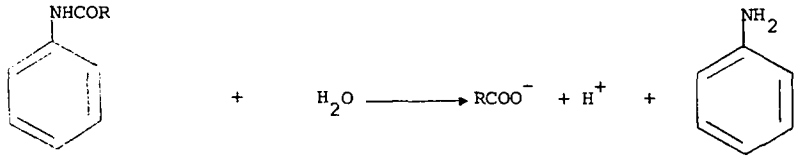
UV analysis of the supernatant solutions after manometric experiments had been completed indicated a complete loss of 4-ethoxyacetanilide (phenacetin), 3-hydroxyacetanilide (metacetamol) and 4-hydroxyacetanilide (paracetamol) from the solution coupled with a characteristic shift in spectrum to 4-ethoxyaniline, 3-aminophenol and 4-aminophenol, respectively. Concentrations of 4-aminophenol and 4-ethoxyaniline remained unchanged after manometric studies with

these compounds in the respirometer, but the concentration of 3-aminophenol decreased indicating metabolism by the *Penicillium* species.

Isolation and identification of degradation products during the metabolism of some acetanilides by the Penicillium species. TLC analysis of the growth medium after incubation of the *Penicillium* species with paracetamol, metacetamol or phenacetin indicated degradation of these compounds to 4-aminophenol, 3-aminophenol and 4-ethoxyaniline, respectively. Isolation and identification of 4-ethoxyaniline as its hydrochloride from phenacetin metabolism was also determined. TLC analysis also indicated that 3-aminophenol was slowly metabolised by the mould.

DISCUSSION AND CONCLUSIONS

The enzymatic hydrolysis of N-arylacylamides is as follows:



The fatty acid anion liberated is further metabolised by enzymatic oxidation whilst the arylamine is not degraded but accumulates in the medium. Degradation of N-(3,4-dichlorophenyl)–2-methyl pentanamide by species of *Penicillium* and *Pullularia* resulted in the production of 2-methyl-valeric acid which was further metabolised and dichloroaniline which accumulates in the growth medium (Sharabi and Bordeleau, 1969). Similarly degradation of a variety of substituted acetanilides by *Corynebacterium pseudodiphtheriticum* resulted in the formation of acetate and the corresponding amine (Grant and Wilson, 1973). The enzyme initially responsible for such degradations is aryl-acylamide amidohydrolase (aryl acylamidase EC 3.5.1.13) and has been isolated and purified from *Penicillium* species by Sharabi and Bordeleau (1969).

Studies with the *Penicillium* species isolated and used by us indicated that a similar degradative pathway is involved for the substituted acetanilides, paracetamol and phenacetin, since the accumulation of 4-aminophenol and 4-ethoxyaniline, respectively, were detected from the metabolism of these compounds. However, metacetamol and its degradation product (3-aminophenol) was apparently metabolised beyond the amine state. These studies indicate that the *Penicillium* species isolated from a solution of paracetamol is capable of growth with, and the hydrolysis of, a series of substituted acetanilides and related compounds and therefore possesses the enzyme, aryl acylamidase.

Utilization for growth, as sole carbon sources, of acetamide and other monocarboxylic acid amides indicates the possession of the enzyme acylamide amidohydrolase (amidase EC 3.5.1.4) by the mould. Similarly, since N-acetylglycine is utilised for growth by the mould, presence of N-acylaminoacid amidohydrolase (aminoacylase EC 3.5.1.14) is also indicated.

In conclusion therefore the *Penicillium* species isolated is another example of the many microorganisms present in the environment which are capable of utilising for growth and subsequently metabolising a variety of compounds from simple phenols to the more complex molecules used as herbicides.

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