

# Formation of 6-Aminopenicillanic Acid, Penicillins, and Penicillin Acylase by Various Fungi

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Received for publication 20 August 1965

## ABSTRACT

COLE, M. (Beecham Research Laboratories, Brockham Park, Betchworth, Surrey, England). Formation of 6-aminopenicillanic acid, penicillins, and penicillin acylase by various fungi. *Appl. Microbiol.* **14**:98-104. 1966.—Several penicillin-producing fungi were examined for ability to produce 6-aminopenicillanic acid (6-APA) and penicillin acylase. 6-APA was found in corn steep liquor fermentations of *Trichophyton mentagrophytes*, *Aspergillus ochraceus*, and three strains of *Penicillium* sp. 6-APA was not detected in fermentations of *Epidermophyton floccosum* although penicillins were produced. 6-APA formed a large part of the total antibiotic production of *T. mentagrophytes*. The types of penicillins produced by various fungi were identified by paper chromatography, and it was found that all cultures produced benzylpenicillin. *T. mentagrophytes* and *A. ochraceus* showed increased yields of benzylpenicillin and the formation of phenoxymethylpenicillin in response to the addition to the fermentation medium of phenylacetic acid and phenoxyacetic acid, respectively. Washed mycelia of the three *Penicillium* spp. and two high penicillin-yielding strains of *P. chrysogenum* possessed penicillin acylase activity against phenoxymethylpenicillin. *A. ochraceus*, *T. mentagrophytes*, *E. floccosum*, and *Cephalosporium* sp. also had penicillin acylase activity against phenoxymethylpenicillin. Only two of the above fungi, *T. mentagrophytes* and *E. floccosum*, showed significant penicillin acylase activity against benzylpenicillin; in both cases it was very low. The acylase activity of *A. ochraceus* was considerably increased by culturing in the presence of phenoxyacetic acid. It is concluded that 6-APA frequently but not invariably accompanies the formation of penicillin, and that penicillin acylase activity against phenoxymethylpenicillin is present in all penicillin-producing fungi.

Penicillin production has been shown to be accompanied by the formation of 6-aminopenicillanic acid (6-APA), not only with strains of *Penicillium chrysogenum* (4), but also with *Cephalosporium* and *Emericellopsis* (8). The formation of penicillin, however, is not confined to these fungi but has also been reported for other species of *Penicillium* and for species of *Aspergillus* (15) and certain dermatophytes (13, 16, 17). Three dermatophyte cultures (two strains of *Trichophyton mentagrophytes* and one strain of *Epidermophyton floccosum*) and an *Aspergillus* culture (*A. ochraceus*) which the author had found to produce penicillin were therefore examined for the production of 6-APA. Some penicillin-producing cultures of *Penicillium* spp. (wild types isolated from various sources) were similarly examined. A typical penicillin-producing strain of *P. chrysogenum* was used for the purpose

of comparison. Since carrying out the investigation, Uri, Valu, and Békési (18) have reported the presence of small amounts of a substance, closely resembling 6-APA, in fermentations carried out with species of both *Epidermophyton* and *Trichophyton*.

An attempt was made to identify the various types of penicillin produced by the fungi which were being examined for 6-APA formation. Certain of these fungi were also examined for the presence of the penicillin acylase enzyme, and tests were conducted to determine whether phenylacetic and phenoxyacetic acids would stimulate the production of benzylpenicillin and phenoxymethylpenicillin, respectively. Phenoxyacetic acid was also tested as an inducer of penicillin acylase in view of the recent work by Uri, Valu, and Békési (19).

## MATERIALS AND METHODS

**Fermentation media and cultural conditions.** A corn steep liquor-lactose medium (medium 1) of the following composition was found to be suitable for the production of 6-APA and penicillins: corn steep liquor (about 50% solids, Brown and Polson Ltd., London, England), 6% (v/v); lactose, 5% (w/v); glucose, 0.5%; NaNO<sub>3</sub>, 0.5%; KH<sub>2</sub>PO<sub>4</sub>, 0.1%; Na<sub>2</sub>SO<sub>4</sub>, 0.1%; CaCO<sub>3</sub>, 1.0%. In some experiments, sodium phenoxyacetate was included in this medium (final concentration, 0.1%, in terms of free acid) to test the effect of this substance on the penicillin acylase activity of the mycelium. The medium was adjusted to pH 4.8 before autoclaving and was dispensed in 100-ml volumes in 500-ml conical flasks closed with cotton wool plugs. After inoculation with a spore suspension obtained from a glycerol-molasses-agar slope, the flasks were shaken for 4 to 6 days at 26 C and 280 rev/min on a rotary shaker with 2-inch amplitude. To obtain good growth for the dermatophytes, it was sometimes necessary to use a seed stage consisting of the same medium and conditions as the fermentation stage, 10 ml being used to inoculate the fermentation stage. The glycerol-molasses-agar had the following composition: glycerol, 0.75% (w/v); molasses, 0.25% (w/v); yeast extract, 0.1% (w/v); MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.005%; KH<sub>2</sub>PO<sub>4</sub>, 0.006%; peptone, 0.5%; NaCl, 2.0%; FeSO<sub>4</sub>, 0.0003%; CuSO<sub>4</sub>, 0.0001%; agar, 2%. The medium was made up in tap water without pH adjustment. Improved yields of 6-APA and penicillins were obtained for *A. ochraceous* BRL 731 when the following medium (medium 2) was used: corn steep liquor (as above), 5% (v/v); lactose, 4% (w/v); glucose, 1%; Na<sub>2</sub>HPO<sub>4</sub>, 0.5%; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.025%; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01%; CaCl<sub>2</sub>, 0.01%; CaCO<sub>3</sub>, 0.25%, and three drops of antifoam (2% octadecanol in cotton seed oil). This antifoam was also added during the fermentation if necessary. The medium was adjusted to pH 6 before autoclaving and was dispensed in 100-ml volumes in 500-ml conical flasks having internal indentations (three equally spaced indentations 1 cm deep and 1 cm from the base of the flask) which acted as baffles. The flasks were covered with thin cotton-wool gauze pads (Martindale face mask refills, Martindale Electric Co., London, England). The flasks were inoculated with a spore suspension from a glycerol-molasses-agar slope and were shaken for 4 to 6 days on a rotary shaker at 26 C, as above.

**Paper chromatography of 6-APA and penicillins.** Whatman no. 1 paper chromatograms 1 cm wide were loaded with two or more superimposed spots of culture filtrate, each spot being 7  $\mu$ liters. 6-APA was separated from penicillins by use of the solvents *n*-butanol-pyridine-water, 1:1:1 (v/v), or *n*-butanol-ethyl alcohol-water, 4:1:5 (v/v), top phase. After developing duplicate chromatograms, 6-APA was rendered biologically active on one of the pair by phenylacetylation (4, 8). Authentic 6-APA prepared by enzymatic deacylation of benzylpenicillin was used as a marker. The various penicillins were separated by use of the two systems already mentioned and the water-saturated ether system described by

Karnovsky and Johnson (9) with the paper buffered at pH 6.2. When the ether system was used, the penicillins were first concentrated by extracting to avoid loading the ether chromatograms with unwanted solids. The extracts were prepared by chilling culture filtrates, adjusting the pH to 2, extracting with 0.25 volume of *n*-butyl acetate, and back-extracting the separated butyl acetate with 0.5 volume of 0.5% NaHCO<sub>3</sub>. Two spots of these solutions were then applied to the chromatogram. The various penicillin zones were identified by comparison with a benzylpenicillin marker (Glaxo Crystapen) and a marker consisting of a culture filtrate of a typical penicillin-producing strain of *P. chrysogenum* for which the various penicillin zones are well known (9). All chromatograms were contacted with agar seeded with *Bacillus subtilis* to locate the position of the various antibiotic substances.

**Biological assays.** The agar plate diffusion assay with *B. subtilis* as seed organism was used for determination of levels of penicillin in culture filtrates.

**Stability to penicillinase.** The penicillinase consisted of the cell-free culture fluid of *B. cereus* prepared as described by Batchelor et al. (2). Samples of cell-free fermentation broth or extracted penicillin material were incubated for 15 min at 37 C and pH 7 with penicillinase at a final concentration of 1%. The samples were then examined for biological activity as in biological assays or were examined by paper chromatography.

**Stability to acid.** Samples of the cell-free fermentation broth or extracted penicillins were adjusted to pH 2 and incubated at 37 C for 10 or 20 min before returning the pH to 7 and examining by paper chromatography.

**Carboxylic acids as precursors.** Solutions (5%, pH 7) of phenylacetic acid, phenoxyacetic acid,  $\alpha$ -aminophenylacetic acid, and 2,6-dimethoxybenzoic acid were prepared and sterilized by autoclaving. Fermentations were carried out as described above, and 1 ml of carboxylic acid solution was added at 2 and 3 days to each flask to a final concentration of 0.1%. Control fermentations had no precursor additions.

**Penicillin acylase activity.** Mycelium was obtained by centrifuging 4- to 5-day fermentations in the corn steep liquor-lactose medium with and without 0.1% phenoxyacetic acid as inducer. This mycelium was washed by resuspending in 1 volume of water and recentrifuging. For the high-yielding strains of *P. chrysogenum*, the mycelium was given a further wash. Between 2- and 4-g batches of wet mycelium were mixed with 10-ml volumes of 5 mg/ml solutions of benzyl and phenoxymethylpenicillins in 0.05 M phosphate buffer at pH 8.5 and were incubated at 37 C for 3 hr. Controls consisted of washed mycelium alone in buffer at pH 8.5 and penicillins alone in buffer at pH 8.5. Reaction mixtures were examined for 6-APA by paper chromatography by use of the butanol-ethanol-water system with and without phenylacetylation. Estimations of the yields of 6-APA were made from chromatograms of a range of standard amounts of 6-APA.

TABLE 1. 6-Aminopenicillanic acid and penicillins in shake flask fermentations of various fungi

Organism	Culture filtrate*		Solvent extract of culture filtrate*					
	6-Amino penicillanic acid zone ( $R_F$ 0.48)	Main penicillin zone ( $R_F$ 0.75)	Very slow-moving penicillins (0-1 cm)†	Unidentified penicillins (3-5 cm)	Penicillin G (9 cm)	Penicillin F (14.5 cm)	Dihydro penicillin F (19 cm)	Penicillin K (26-33 cm)
<i>Penicillium</i> sp. BRL 733.....	0	20	15	0	21	19	0	0
<i>Penicillium</i> sp. BRL 735.....	17	26	22	14	22	24	21	21
<i>Penicillium</i> sp. BRL 737.....	16	18	18	Trace	20	16	16	16
<i>P. chrysogenum</i> BRL 781.....	27	23	21	Trace	22	21	19	21
<i>Aspergillus ochraceous</i> BRL 731..	17	24	12	11	19	13	16	0
<i>Trichophyton mentagrophytes</i> BRL 569.....	17	17	18	12	17	15	25	Trace
<i>T. mentagrophytes</i> BRL 579....	20	11	13	Trace	12	Trace	20	0
<i>Epidermophyton floccosum</i> BRL 623.....	0	10	Trace	0	12	14	14	0

\* Results are expressed as the width (in millimeters) of zones on bioautograms. For the culture filtrate, the solvent system was *n*-butanol-pyridine-water (1:1:1, v/v), 14  $\mu$ liters per chromatogram for all cultures except BRL 731 which had 35  $\mu$ liters. For the solvent extract of the culture filtrate, ether saturated with water (pH 6.2), 14  $\mu$ liters per chromatogram, was used.

† Migrations.

## RESULTS

Culture filtrates of 4- or 5-day fermentations of each of the fungi listed in Table 1 were shown to contain penicillins by demonstration of complete loss of biological activity after treatment of samples with penicillinase. All but two of the culture filtrates were also shown to contain a 6-APA-like material by demonstrating the appearance of a biologically active zone in the same position as the 6-APA marker when paper chromatograms were sprayed with phenylacetyl chloride before being contacted with seeded agar. The butanol-pyridine chromatograms of the culture filtrate of *T. mentagrophytes* BRL 579 also showed at  $R_F$  0.3 a trace zone of another 6-APA-like compound which only became biologically active after phenylacetylation. This compound may be the product of a reaction between 6-APA and carbohydrate and may be similar to that found in *P. chrysogenum* fermentations (11). A similar zone was sometimes observed with *A. ochraceous* BRL 731.

The results in Table 1 show that most of the cultures produce a mixture of penicillins similar to those produced by *P. chrysogenum*. However, the dermatophytes and *A. ochraceous* BRL 731 produced little or no penicillin K and *Penicillium* sp. BRL 733 produced very little dihydropenicillin F or penicillin K. *A. ochraceous* BRL 731, *T. mentagrophytes* BRL 569, and *P. chrysogenum* BRL 781 produced a zone corresponding in position to methylpenicillin at  $R_F$  0.58 on the butanol-pyridine chromatograms. The three penicillia and *P. chrysogenum* showed a zone in

the position of penicillin X at  $R_F$  0.3 on butanol-ethyl alcohol chromatograms, culture BRL 735 having the largest zone. *T. mentagrophytes* BRL 569 also showed a trace zone in this position and had a zone at  $R_F$  0.2. In the butanol-ethyl alcohol system, the main penicillin zone was at  $R_F$  0.4 to 0.5.

Mycelium from three of the cultures (*Penicillium* spp. BRL 735 and BRL 737, and *T. mentagrophytes* BRL 569) was extracted with 70% aqueous acetone for 10 min at room temperature by use of 4 g of mycelium and 40 ml of aqueous acetone. When examined by chromatography, all three extracts were found to contain the 6-APA-like compound and penicillins, the extract of BRL 737 containing far less 6-APA-like compound than the others.

The 6-APA-like substances produced by five of the cultures were subjected to a series of tests listed below in which they were found to behave in exactly the same way as authentic 6-APA. These tests were carried out on culture filtrates from 4- to 5-day fermentations, and in the first three tests the 6-APA-like compound was detected by phenylacetylation of chromatograms. (i) Treatment with *B. cereus* penicillinase inactivated the compound. (ii) Treatment at pH 2 for 20 min had little effect on the compound, whereas the penicillins present in the sample were destroyed in 10 min. (iii) Extraction at pH 2 by use of various solvents did not extract the compound from the aqueous phase. (iv) In this test, both the 6-APA-like compound and authentic 6-APA were converted into penicillins

TABLE 2. *Penicillin acylase activity of the mycelium of various fungi and the effect of phenoxyacetic acid on the formation of the enzyme*

Organism	Presence or absence of 0.1% phenoxyacetic acid in corn steep liquor-lactose fermentation medium	Enzyme activity of mycelium after 4 days of growth ( $\mu\text{g/ml}$ of 6-aminopenicillanic acid liberated in 3 hr)*		
		Control mycelium + phosphate buffer	Mycelium + benzyl penicillin	Mycelium + phenoxymethylpenicillin
<i>Penicillium</i> sp. BRL 733	—	0	Trace	7.8
	+	0	Trace	12.5
<i>Penicillium</i> sp. BRL 736	—	0	<4.0	10.0
	+	0	<2.0	7.5
<i>Penicillium</i> sp. BRL 737	—	Trace	<1.5	13.5
	+	<1.0	1.0	24.0
<i>P. chrysogenum</i> BRL 781	—	42.0	40.0	94.0
	+	16.0	23.5	66.0
<i>P. chrysogenum</i> BRL 803	—	11.5	12.5	47.0
	+	12.5	11.0	36.0
<i>Aspergillus ochraceus</i> BRL 731	—	Trace	<1.0	19.0
	+	<1.0	<1.0	130.0
<i>Trichophyton mentagrophytes</i> BRL 569	—	1.5	3.1	23.5
	+	<1.0	1.9	36.0
<i>Epidermophyton floccosum</i> BRL 772	—	0	1.4	53.0
	+	0	1.0	38.0
<i>Cephalosporium</i> sp. CMI 49137	—	0	<1.0	15.8
	+	0	<1.0	15.0
6-APA formed as a result of incubating 10 ml of 5 mg/ml penicillin in 0.05 M phosphate buffer at pH 8.5 and 37 C.			0	2.1

\* Approximately 4 g of washed mycelium plus 10 ml of sodium phenoxymethylpenicillin or potassium benzylpenicillin (5 mg/ml) in 0.05 M phosphate buffer (pH 8.5) at 37 C.

whose properties were compared. Culture filtrate was chilled, adjusted to pH 2, and extracted twice with one-third volume of methyl isobutyl ketone to remove the naturally occurring penicillins. The aqueous phase still at pH 2 was divided into two lots of about 15 ml; one was acylated by shaking for 10 to 15 min with 5 ml of 0.05% phenylacetyl chloride in methyl isobutyl ketone, and the other was shaken for 10 to 15 min with 5 ml of 0.05%  $\alpha$ -phenoxypropionyl chloride in methyl isobutyl ketone. The solvent phases were collected, and the penicillin was extracted by shaking with 5 ml of 0.5% aqueous sodium bicarbonate. The bicarbonate solutions were examined by ether chromatography before and after treating samples for 10 min at pH 2 and 37 C and after treating samples with 1% penicillinase for 10 min at 37 C. When phenylacetyl chloride was used, both the 6-APA and 6-APA-like substances gave a zone in the position of benzylpenicillin which was both acid- and penicillinase-labile. When  $\alpha$ -phenoxypropionyl chloride was used, both 6-APA and 6-APA-like substances gave a zone in the position of  $\alpha$ -phenoxyethylpenicillin which was penicillinase-labile but acid-stable.

An examination was made of the effect of certain carboxylic acids on fermentations with *T. mentagrophytes* BRL 569 (medium 1) and *A. ochraceus* BRL 731 (medium 2). For both fungi, phenylacetic acid and phenoxyacetic acid stimulated production of penicillin but the other acids had no effect. In the case of *T. mentagrophytes* BRL 569, the control yielded 2.3  $\mu\text{g/ml}$  of penicillin, whereas in the presence of phenoxyacetic acid and phenylacetic acid the yields were 8.0 and 14.0  $\mu\text{g/ml}$ , respectively, when assayed in terms of potassium benzylpenicillin. The diameter of the benzylpenicillin zone on the ether chromatogram of the control was increased from 15 to 29 mm when phenylacetic acid was added to the fermentation, and the penicillin F zone of the control, being in a similar position to phenoxymethylpenicillin, was increased from 20 to 29 mm when phenoxyacetic acid was added to the fermentation. The 6-APA-like compound was detected in the control and in fermentations containing the acids. Very similar results were obtained with *A. ochraceus* BRL 731.

The mycelium of each of the nine fungi listed in Table 2 was examined for ability to hydrolyze benzylpenicillin and phenoxymethylpenicillin. It

was found that all nine cultures produced 6-APA from phenoxymethylpenicillin and two, *T. mentagrophytes* and *E. floccosum*, produced 6-APA from benzylpenicillin when allowance was made for the 6-APA production in the controls. Four cultures, *Penicillium* spp. BRL 733 and BRL 737, *A. ochraceous*, and *T. mentagrophytes* BRL 569, had higher acylase activity against phenoxymethylpenicillin after culturing in the presence of phenoxyacetic acid; one culture, *A. ochraceous*, showed a marked increase in activity. Acylase activity against benzylpenicillin was not increased by culturing in the presence of phenoxyacetic acid.

#### DISCUSSION

The results of the present investigation show that penicillin production is accompanied by 6-APA in the two strains of *T. mentagrophytes* examined, with the 6-APA often representing a high proportion of the total antibiotic material. 6-APA was not detected in fermentations of *E. floccosum* BRL 623, but this may have been associated with the fact that the yield of penicillin was very low for this culture. Six other isolates of *E. floccosum* have more recently been tested, but none of the cultures produced detectable amounts of 6-APA. 6-APA also accompanied the penicillins in *A. ochraceous* and in two of the three strains of *Penicillium* sp. For these cultures and the dermatophytes, the yield of 6-APA was not thought to exceed 1 to 2  $\mu\text{g}/\text{ml}$ . The third *Penicillium* strain, BRL 733, produced sufficient penicillin to make it readily detectable, but no trace of 6-APA-like substance was found. It would thus appear that 6-APA does not invariably accompany penicillin in penicillin-producing moulds. The finding of 6-APA in *Trichophyton* fermentations is in agreement with the results described by Uri et al. (18), who also detected 6-APA in *Epidermophyton* fermentations.

The penicillin pattern obtained on ether chromatograms was, for all of the fungi examined, remarkably similar to that for *P. chrysogenum*. All fungi produced hydrophilic penicillin which remained at the origin, all produced benzylpenicillin and penicillin F, and all but one produced dihydro penicillin F. These results are in marked contrast to the pattern for *Cephalosporium* sp. and *Emericellopsis* sp., which also produce penicillins. It is interesting to note that penicillin K is not a major component of the penicillin mixture produced by the dermatophytes or the *A. ochraceous*, and is not always produced by *Penicillium* spp. As in *P. chrysogenum* fermentations, the production of benzylpenicillin and phenoxymethylpenicillin was stimulated by the addition of phenylacetic acid and phenoxyacetic

acid, respectively, to fermentations of *Trichophyton* and *Aspergillus*. Stimulation of penicillin production by phenylacetic acid in *Trichophyton* fermentations has also been observed by Uri et al. (16).

Using *P. chrysogenum*, Murao and Kishida (12), Erickson and Bennett (Bacteriol. Proc., p. 65, 1961), and Claridge, Luttinger, and Lein (6) have reported low levels of penicillin acylase activity when using benzylpenicillin as substrate. Although Erickson and Bennett reported finding no 6-APA production in the absence of added penicillin, results can be confused by the liberation of 6-APA from thoroughly washed *P. chrysogenum* mycelium, a process which can be shown to take place very readily, particularly in strains selected for production of 6-APA and penicillins. In experiments reported in this paper, the amount of 6-APA liberated by the fungal mycelium was determined in the absence as well as in the presence of added benzylpenicillin or phenoxymethylpenicillin. The amount of 6-APA formed on incubating the penicillin alone in buffer was also determined, since phenoxymethylpenicillin has been reported to break down to liberate 6-APA under these conditions (1). Three strains of *Penicillium* sp., two *P. chrysogenum* strains, *T. mentagrophytes* BRL 569, *E. floccosum* BRL 772, *A. ochraceous*, and *Cephalosporium* sp. CMI 49137 were found to have penicillin acylase activity against phenoxymethylpenicillin; however, for all fungi, activity against benzylpenicillin was extremely low or absent, with only two, *T. mentagrophytes* BRL 569 and *E. floccosum* BRL 772, clearly showing activity.

Uri et al. (18) have also reported the presence of penicillin acylase activity in all of the dermatophyte cultures they examined, with activity against phenoxymethylpenicillin usually being greater than that against benzylpenicillin; in addition, these workers reported (19) that this enzyme could be induced by phenoxyacetic acid. In the present work, *T. mentagrophytes* BRL 569 was found to show higher acylase activity against phenoxymethylpenicillin after culturing in the presence of phenoxyacetic acid, but the activity of *E. floccosum* BRL 772 was not increased. As phenoxyacetic acid can stimulate the production of phenoxymethylpenicillin in *Trichophyton*, some of this penicillin must be deacylated to 6-APA by the induced penicillin acylase. Penicillin acylase activity against phenoxymethylpenicillin was sometimes increased by culturing other penicillin-producing fungi in the presence of phenoxyacetic acid. *A. ochraceous* showed a large increase in activity, and small increases in activity were obtained with *Penicillium* spp. BRL 733 and BRL 737 and with *T. mentagrophytes* BRL 569.

In no instance did the presence of phenoxyacetic acid in the growth medium increase the acylase activity against benzylpenicillin. If a mould produces a low and variable level of acylase enzyme whose preferred substrate is phenoxymethylpenicillin, the activity against benzylpenicillin may be difficult to demonstrate, and this may explain some of the negative results obtained for various cultures described in this paper and those obtained by Rolinson et al. (14) and Claridge, Gourevitch, and Lein (5) for *P. chrysogenum*.

The penicillin acylase enzyme of *E. coli* is reversible, is specific for the acyl side chain of penicillin, and can remove this acyl group from or attach it to a variety of amino acids (7, 10). If the same is true for the penicillin biosynthesis enzymes of *P. chrysogenum*, then the acylases involved in attaching acids such as phenylacetic acid and phenoxyacetic acid to some intermediate amino acid might also be expected to be able to remove these acyl groups from penicillins under suitable conditions. However, when benzylpenicillin was exposed to the mycelium of a high-yielding strain of *P. chrysogenum*, no penicillin deacylase was observed, and when similar mycelium was exposed to phenoxymethylpenicillin only low deacylase activity was observed. Penicillins would not be expected to accumulate in a *P. chrysogenum* fermentation if the mould produced significant amounts of extracellular deacylase enzyme and if the extracellular conditions were favorable for its activity. The results for *P. chrysogenum* might be explained by the penicillin acylase enzymes being intracellular, the substrate not being able to gain access to the enzyme because of the permeability barrier, and the enzymes being in an environment within the cell which was more suitable for acylation than for deacylation. Accordingly, the various penicillin-producing fungi which show penicillin deacylase activity may do so because of the presence of small amounts of extracellular acylase, some of which is attached to hyphal cell walls. As phenoxymethylpenicillin is hydrolyzed more readily than benzylpenicillin by the cultures mentioned in this paper, it is suggested that the main extracellular enzyme is a phenoxymethylpenicillin acylase. At least one of the cultures, namely, *T. mentagrophytes* BRL 569, had significant benzylpenicillin acylase activity, produced benzylpenicillin, and had fermentations of pH 8 or higher. It would seem from this that at least some of the 6-APA which was produced could have arisen by extracellular deacylation of benzylpenicillin.

In the present investigation, all of the penicillin-producing fungi examined for phenoxymethylpenicillin acylase activity were found to produce

the enzyme. Data also exist for various other strains of *P. chrysogenum* (6), and for *Emerickia minima* (Stolk) (8), *Cephalosporium* sp. (6), and dermatophytes (18). However, the property of producing phenoxymethylpenicillin acylase is widespread among moulds (3), and is not always accompanied by penicillin production. In this connection, it is of interest to note that in unpublished experiments carried out in the author's laboratory penicillin formation was demonstrated when 6-APA was added to a fermentation containing a mould which did not otherwise produce penicillin. In these experiments with an unidentified culture of *Penicillium* (BRL 807), benzylpenicillin and other "natural penicillins" were produced. In similar experiments, a yeast (BRL 809) was found to produce a single penicillin resembling methylpenicillin in  $R_F$  value. The most likely explanation for these results is that a penicillin acylase was bringing about the coupling to 6-APA of carboxylic acids present in the medium. Phenoxymethylpenicillin was produced when phenoxyacetic acid and 6-APA were added to the *Penicillium* fermentation, and the benzylpenicillin yield was enhanced by adding phenylacetic acid along with the 6-APA. The formation of methylpenicillin from 6-APA by the yeast suggests that a third type of penicillin acylase (methylpenicillin acylase) exists in addition to the phenoxymethylpenicillin and benzylpenicillin acylases.

#### ACKNOWLEDGMENTS

I am indebted to Mrs. M. V. Hart for technical assistance, to G. N. Rolinson for advice and encouragement, and to M. Richards for identifying the *A. ochraceous* and supplying the dermatophytes.

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