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

M. COLE
Research Division, Beecham Research Laboratories, Brockham Park, Betchworth, Surrey, England

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 ochraceous, 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. ochraceous showed increased yields of benzylpenicillin and the formation of phenoxyethylpenicillin 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 phenoxyethylpenicillin. A. ochraceous, T. mentagrophytes, E. floccosum, and Cephalosporium sp. also had penicillin acylase activity against phenoxyethylpenicillin. 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. ochraceous 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 phenoxyethylpenicillin 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. ochraceous) 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 phenylacetid and phenoxacetic acids would stimulate the production of benzylpenicillin and phenoxyethylpenicillin, respectively. Phenoxyacetid acid was also tested as an inducer of penicillin acylase in view of the recent work by Uri, Valu, and Békési (19).
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%; NaNO₃, 0.5%; KH₂PO₄, 0.1%; Na₂SO₄, 0.1%; CaCO₃, 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); MgSO₄·7H₂O, 0.005%; KH₂PO₄, 0.006%; peptone, 0.5%; NaCl, 2.0%; FeSO₄·0.003%; CuSO₄·0.0001%; agar, 2%. The medium was made up in tap water without pH adjustment. Improved yields of 6-APA and penicillins were obtained for A. oryzae BRL 731 when the following medium (medium 2) was used: corn steep liquor (as above), 5% (v/v); lactose, 4% (w/v); glucose, 1%; NaH₂PO₄, 0.5%; MnSO₄·H₂O, 0.025%; FeSO₄·7H₂O, 0.01%; CaCl₂, 0.01%; CaCO₃, 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 μlitters. 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 deacetylation 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% NaHCO₃. Two spots of these solutions were then applied to the chromatogram. The various penicillin zones were identified by comparison with a benzylpenicillin marker (Glaxo Crystopen) 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, α-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% phenoxycetic 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 phenoxyethylpenicillins 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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Culture filtrate*</th>
<th>Solvent extract of culture filtrate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-Amino penicillanic acid zone (RF 0.48)</td>
<td>Very slow-moving penicillins (0-1 cm)†</td>
</tr>
<tr>
<td></td>
<td>Main penicillin zone (RF 0.75)</td>
<td>Unidentified penicillins (3-5 cm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Penicillin G (9 cm)</td>
</tr>
<tr>
<td>Penicillium sp. BRL 733</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Penicillium sp. BRL 735</td>
<td>17</td>
<td>26</td>
</tr>
<tr>
<td>Penicillium sp. BRL 737</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>P. chrysogenum BRL 781</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>Aspergillus ochraceous BRL 731</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>Trichophyton mentagrophytes BRL 569</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>T. mentagrophytes BRL 579</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Epidermophyton floccosum BRL 623</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

* 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 µl per chromatogram for all cultures except BRL 731 which had 35 µl. For the solvent extract of the culture filtrate, ether saturated with water (pH 6.2), 14 µl 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 RF 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 RF 0.58 on the butanol-pyridine chromatograms. The three penicillia and P. chrysogenum showed a zone in the position of penicillin X at RF 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 RF 0.2. In the butanol-ethyl alcohol system, the main penicillin zone was at RF 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.
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 acetylated by shaking for 10 to 15 min with 5 ml of 0.05% phenylacetic chloride in methyl isobutyl ketone, and the other was shaken for 10 to 15 min with 5 ml of 0.05% α-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 phenylacetic 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 α-phenoxypropionyl chloride was used, both 6-APA and 6-APA-like substances gave a zone in the position of α-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. ochraceous* 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 µg/ml of penicillin, whereas in the presence of phenoxyacetic acid and phenylacetic acid the yields were 8.0 and 14.0 µg/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 phenoxyethylpenicillin, 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. ochraceous* BRL 731.

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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Presence or absence of 0.1% phenoxyacetic acid in corn steep liquor-lactose fermentation medium</th>
<th>Enzyme activity of mycelium after 4 days of growth (µg/ml of 6-aminopenicillanic acid liberated in 3 hr)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control mycelium + phosphate buffer</td>
</tr>
<tr>
<td><strong>Penicillium sp. BRL 733</strong></td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td><strong>Penicillium sp. BRL 736</strong></td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td><strong>Penicillium sp. BRL 737</strong></td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td><strong>Penicillium sp. BRL 737</strong></td>
<td>-</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td><strong>P. chrysogenum BRL 781</strong></td>
<td>-</td>
<td>42.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>16.0</td>
</tr>
<tr>
<td><strong>P. chrysogenum BRL 803</strong></td>
<td>-</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>12.5</td>
</tr>
<tr>
<td><strong>Aspergillus ochraceous BRL 731</strong></td>
<td>-</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td><strong>Trichophyton mentagrophytes BRL 569</strong></td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td><strong>Epidermophyton floccosum BRL 772</strong></td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td><strong>Cephalosporium sp. CMI 49137</strong></td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

* *Approximately 4 g of washed mycelium plus 10 ml of sodium phenoxyoxymethylpenicillin or potassium benzylpenicillin (5 mg/ml) in 0.05 M phosphate buffer at pH 8.5 and 37 C.*
was found that all nine cultures produced 6-APA from phenoxyacetymethylpenicillin and two, T. mента-
grophytes 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 phenoxy-
methylpenicillin 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 peni-
cillins 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 µg/ml. The third Peni-
cillium 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 in-
varily accompany penicillin in penicillin-pro-
ducing moulds. The finding of 6-APA in Tricho-
phyton fermentations is in agreement with the results described by Uri et al. (18), who also de-
tected 6-APA in Epidermophyton fermentations.

The penicillin pattern obtained on ether chro-
matograms 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 benzyl-
penicillin and penicillin F, and all but one pro-
duced dihydro penicillin F. These results are in
marked contrast to the pattern for Cephalo-
sporium sp. and Emereccellipsis sp., which also pro-
duce 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 fer-
mentations, the production of benzylpenicillin and phenoxyacetymethylpenicillin was stimulated by the addition of phenylacetic acid and phenoxyacetic acid, respectively, to fermentations of Trichophy-
ton and Aspergillus. Stimulation of penicillin produ-
cion 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 ac-
tivity 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 liber-
aton 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 phenoxyacetymethylpenicillin. The amount of 6-APA formed on incubating the penicillin alone in buffer was also determined, since phenoxyacetymethylpenicillin 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 phenoxyacetymethylpenicillin; how-
ever, for all fungi, activity against benzylpeni-
cillin 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 dermatophytes cultures they examined, with activity against phenoxyacetymethylpenicillin usually being greater than that against benzylpenicillin; in addition, these workers reported (19) that the enzyme could be induced by phenoxyacetic acid. In the present work, T. mentagrophytes BRL 569 was found to show higher acylase activity against phenoxyacetymethylpenicillin 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 phenoxyacetymethylpenicillin in Trichophyton, some of this penicillin must be decacylated to 6-APA by the induced penicillin acylase. Penicillin acylase activity against phenoxyacetymethylpenicillin 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 phenoxyethylpenicillin, 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 phenoxyethylpenicillin 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 phenoxyethylpenicillin is hydrolyzed more readily than benzylpenicillin by the cultures mentioned in this paper, it is suggested that the main extracellular enzyme is a phenoxyethylpenicillin 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 phenoxyethylpenicillin acylase activity were found to produce the enzyme. Data also exist for various other strains of _P. chrysogenum_ (6), and for _Emerecellopsis minima_ (Stolk) (8), _Cephalosporium_ sp. (6), and dermatophytes (18). However, the property of producing phenoxyethylpenicillin 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_ 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. Phenoxyethylpenicillin 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 phenoxyethylpenicillin 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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