Effect of Pentachloronitrobenzene (PCNB)
and Other Chemicals on Sensitive and PCNB-resistant Strains of
Aspergillus nidulans

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SUMMARY
Pentachloronitrobenzene (PCNB)-sensitive and PCNB-resistant strains of
Aspergillus nidulans were grown in liquid and on solid media with and without
PCNB. On solid medium with PCNB, hyphae of the sensitive strain had thick
walls, were shorter, branched less and had fewer nuclei per cell compared with the
control. Under these conditions resistant hyphae were normal and less affected in
growth parameters. In liquid medium the effect of PCNB on growth was less marked,
but production of extracellular polysaccharide was decreased in the sensitive strain
but not in the resistant one. The walls of resistant hyphae had a low hexosamine
content compared with those of the wild-type; it increased in both by about a
third in the presence of PCNB. Sodium deoxycholate had no effect on it. There was
no difference in alkali-soluble material between the strains; it was decreased in both
in the presence of PCNB, but more so in the sensitive than in the resistant strain.
Changes in carbohydrate synthesis were a result of PCNB action on the sensitive
wild-type; the resistant mutant withstood similar changes without such gross
changes in morphology.

INTRODUCTION
Pentachloronitrobenzene (PCNB) and tetrachloronitrobenzene (TCNB) restrict the
growth and conidiation of sensitive but not resistant strains of Aspergillus nidulans (Threl-
fall, 1968). Resistance is controlled by either of two closely linked genes on chromosome III
but how they control resistance is unknown.

Some fungi, for example Pythium and Phytophthora, are insensitive to PCNB (Reavill,
1954) and the observation that these fungi have cellulose in the hyphal wall (Frey, 1950)
may be important in determining their susceptibility. Many chemicals can induce marked
changes in the growth and morphology of fungi. Sorbose induces a colonial growth habit
in Neurospora crassa (de Terra & Tatum, 1961) and increases the glucosamine and decreases
the glucose content of its walls. Makris & Georgopoulos (1969) have shown that the
hexosamine content of hyphal walls of this fungus is decreased in the presence of PCNB.
Permanent morphogenetic changes can be brought about by mutation. In N. crassa a
colonial mutant (col-2) has an altered glucose-6-dehydrogenase which results in the accumu-
lation of glucose 6-phosphate. This enzyme is normal in the sorbose-induced phenocopy
(Trevithick & Metzenberg, 1966).

This paper describes changes in the cell morphology and chemical composition of walls
of Aspergillus nidulans strains, resistant and sensitive to PCNB when grown in the presence
of this and other chemicals.
METHODS

The general methods, media and chemicals used have been described (Threlfall, 1968). Most of the work has been carried out with the Glasgow wild-type strain (FGSC 4) and a mutant (pcnb-2) resistant to PCNB.

**Growth of cultures in liquid medium.** Cultures were grown for 6 days at 37 °C in 200 ml liquid complete medium (CM) in 500 ml conical flasks on a rotary shaker at 150 rev./min. If required PCNB or acenaphthene dissolved in acetone was added to give a final concentration of 125 μg/ml and 50 μg/ml respectively. Acetone alone had no effect on the growth of cultures. Sodium deoxycholate dissolved in water was added to give a final concentration of 800 μg/ml. Each flask was inoculated with 0·5 ml of a suspension containing 10⁶ conidia/ml.

**Preparation of walls.** The mycelium was collected from liquid cultures by straining through muslin. It was washed several times with warm water, then with cold water and finally with acetone. The mycelium was squeezed between blotting paper and small fragments dropped into liquid nitrogen. The frozen material was ground in a mortar and pestle, cooled with liquid nitrogen. The finely ground mycelium was suspended in distilled water, collected on filter paper, washed with industrial methylated spirit (74 o.p.), acetone, blotted dry, frozen and ground as before. This process was repeated until a uniform fine powder was obtained. The powdered mycelium was suspended in industrial methylated spirit, centrifuged, re-suspended in petroleum ether, again centrifuged and the pellet dried at 37 °C for 2 days. Wall preparations were stored in a desiccator at 4 °C.

**Preparation of extracellular polysaccharide.** To 100 ml culture filtrate was added 100 ml industrial methylated spirit (74 o.p.) and the mixture left overnight at 4 °C. The gelatinous precipitate was collected on filter paper, washed with ethanol, ether and acetone, removed to a tared glass tube, dried in a desiccator and weighed.

**Estimation of wall components.** Estimation of the hexosamine content of hyphal walls was made on material hydrolysed in hydrochloric acid. Samples (30 to 60 mg) were dried overnight in vacuo and weighed. They were hydrolysed in 5 ml 3N-HCl at 105 °C for 18 h in vacuo. The hydrolysates were evaporated to dryness over solid KOH in a desiccator in vacuo. Water was added to the residue, the solution filtered and made up to 100 ml. Hexosamines were determined by the method of Dische & Borenfreund (1950) with glucosamine as standard. Hexoses were assayed in sulphuric acid hydrolysates. To 20 to 40 mg material dried as above was added 1 ml 72% (w/w) sulphuric acid at 4 °C and the mixture gently agitated for 2 h. Water (9 ml) was added and the mixture incubated at 100 °C for 4 h. The mixture was filtered and made up to 100 ml. Hexoses were determined by the method of Dubois et al. (1956) with glucose as standard. Samples (20 to 40 mg) of wall material were extracted in sealed tubes with 10 ml 2 N-NaOH for two periods of 16 h at room temperature. The material was washed several times with water and dried in a desiccator in vacuo. The loss in weight after extraction was calculated.

**Cytological techniques.** Strips of thin sterile Cellophane were placed on the surface of CM agar in Petri dishes and inoculated with a suspension of conidia. If required, 1 mg acenaphthene or 1 mg PCNB was deposited on the inside of the lid of the inverted dish by adding an acetone solution of the chemical.

After incubation the strips were removed and fixed in acetic acid:ethanol (1:3, v/v) for 10 min. The strips were washed in ethanol, 75%, 50%, 25% ethanol and finally in water. To stain nuclei they were treated with N-HCl for 20 to 30 min at room temperature and then with N-HCl for 10 min at 60 °C. After washing in 67 mM-phosphate buffer at pH 6·9 for 5 min they were stained for 3 to 5 h in Giemsa R66 (G. T. Gurr Ltd, Romford, Essex) diluted
**Aspergillus nidulans: cell wall changes**

Fig. 1. *Aspergillus nidulans*: diameters of colonies of wild-type sensitive to pentachloronitrobenzene (PCNB) and *pcnb-2* resistant to PCNB after 6 days at 37°C on complete medium containing 0 to 1000 μg acenaphthene/ml (means of five replicates) — — , wild-type; — O — , *pcnb-2*; and 0 to 1600 μg sodium deoxycholate/ml (means of five replicates) — — , wild-type; — O — , *pcnb-2*.

1:10 with buffer. The number of nuclei in each cell was noted on a drawing made with the aid of a camera lucida. From the drawing the length of hyphae could be calculated. Insoluble polysaccharide was stained by the periodic acid Schiff method. The material fixed as above was brought to water and treated with 0.5% periodic acid for 10 to 20 min. It was washed in running water for 10 min and placed in Feulgen's reagent for 10 to 30 min, rinsed and treated in 2% sodium bisulphite for 1 to 2 min. The strip was then washed in running water for 10 min, dehydrated in 25%, 50%, 75% ethanol and finally pure ethanol, cleared and mounted for examination in clove oil. α1:4- and α1:6-linked glucose units were hydrolysed by treating hyphae or wall material with the solution obtained on adding 5 mg amyloglucosidase (glucoamylase) Grade 3 (Sigma Chemical Co., London, S.W. 6) to 1 ml 0.2 M acetate buffer at pH 4.5. Succinate dehydrogenase activity was detected by incubating unfixed material in a mixture of 2 ml 67 mM-phosphate buffer at pH 6.9, 1 ml sodium succinate and 1 ml 0.1% 2,5-diphenyl-3-(4-styrylphenyl) tetrazolium chloride.

**RESULTS**

*Effect of acenaphthene and sodium deoxycholate on growth and morphology of wild-type and resistant hyphae*

A cenaphthene restricted colony growth of *Aspergillus nidulans* when 1 mg was added to the inside of the lid of an inverted Petri dish culture of the fungus. Sodium deoxycholate is routinely used to restrict the growth of colonies without impeding conidiation (Mackintosh & Pritchard, 1963). The effect of these chemicals on growth of PCNB-sensitive and resistant
strains was investigated. A series of Petri dishes (8.5 cm diameter), each containing 20 ml CM agar and 0, 10, 50, 100, 200, 500, 1000 µg acenaphthene/ml or 0, 10, 50, 100, 200, 400, 800, 1600 µg sodium, deoxycholate/ml was prepared by adding a solution of the chemical to the warm medium before pouring into dishes. Each dish was inoculated at the centre with a disc (2 mm diameter) of mycelium cut from the periphery of a colony growing on CM. Five replicate dishes were set up for each concentration and after 6 days of incubation two diameters at right angles of each colony were measured and the average value calculated. Both strains were about equally restricted by deoxycholate (Fig. 1). Conidiation was normal. The wild-type strain was inhibited by acenaphthene and production of conidia was diminished as the concentration of the inhibitor increased. The resistant strain responded in the same way that it did to deoxycholate but conidia became rather sparse and the colony became fawn coloured at 500 µg and 1000 µg acenaphthene/ml.

Cytology

Hyphae of PCNB-treated sensitive strains were wider and their linear growth slower compared with untreated hyphae. The hyphae of a resistant strain exposed to PCNB could not be visually distinguished from those of a sensitive strain grown in the absence of PCNB. The hyphal wall of a sensitive strain exposed to PCNB was thicker than that of untreated hyphae; the cells were shorter and irregularly swollen with the wall thickest at the widest part of the cell. Table 1 presents measurements made on electron micrographs of transverse sections of hyphae grown on CM agar some of which had been exposed to PCNB vapour. Microcolonies which had developed from single conidia after 14 h were stained with Giemsa and examined. The results in Table 2 show that PCNB severely restricted hyphal growth and nuclear division but had less effect on the branching of hyphae or on the formation of cross walls.

Observation of periodic acid-Schiff-stained preparations showed the presence of many small (about 0.5 µm) dark red bodies in the cytoplasm of control hyphae which could not be seen in hyphae exposed to PCNB. The thickened walls and cross walls of PCNB-treated sensitive strains stained bright red with this treatment but, after colonies had been incubated with amyloglucosidase at 37°C for 22 h and then stained, the wall thickening had disappeared. After enzyme treatment the small bodies in the cytoplasm of control hyphae could not be detected by staining.

Tetrazolium staining showed some cells in PCNB-treated hyphae stained pale green while in other cells in the same hypha the cytoplasm remained unstained. Mitochondria were detected in both control and PCNB-treated cells; they stained pink and were seen to fade on irrigation of the material with 0.16 M-sodium iodoacetate.

The effect of acenaphthene and PCNB on growth of wild-type and resistant strains after 13 h incubation is shown in Table 3. It was noted that resistant hyphae were more sensitive to hydrolysis than the wild-type and best preparations were obtained by treatment for 5 min each with cold and hot HCl prior to staining. Difficulty was experienced in counting nuclei in resistant hyphae because there were many small bodies which stained, the largest of which could be confused with nuclei. Results are not given for the wild-type strain in the presence of acenaphthene because the conidia did not germinate; those of the resistant strain were able to germinate and make some growth. PCNB retarded growth of both resistant and sensitive strains but its effect was most marked on the latter. The low number of nuclei in cells of the resistant strain in the control may not be significant because of the difficulty of counting nuclei in this strain.
Aspergillus nidulans: cell wall changes

Table 1. Aspergillus nidulans: effect of pentachloronitrobenzene (PCNB) vapour on hyphal wall thickness and on hyphal lumen diameter of the wild-type strain

Each value the average of 12 measurements.

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>PCNB treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness of hyphal wall (µm)</td>
<td>0·135</td>
<td>0·200</td>
</tr>
<tr>
<td>Diameter of hyphal lumen (µm)</td>
<td>1·5</td>
<td>1·8</td>
</tr>
</tbody>
</table>

Table 2. Aspergillus nidulans: effect of pentachloronitrobenzene (PCNB) on the number of nuclei, number of hyphal tips and cell size of colonies of the wild-type strain 14 h after inoculation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of colonies</th>
<th>Total cells</th>
<th>Total nuclei</th>
<th>Hyphal length (µm)</th>
<th>Hyphal tips</th>
<th>Nuclei/cell</th>
<th>Cell length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>5</td>
<td>11·80</td>
<td>327·2</td>
<td>5974</td>
<td>19·00</td>
<td>28·9</td>
<td>516·6</td>
</tr>
<tr>
<td>PCNB</td>
<td>8</td>
<td>8·25</td>
<td>33·7</td>
<td>157</td>
<td>7·25</td>
<td>4·1</td>
<td>19·3</td>
</tr>
</tbody>
</table>

Table 3. Aspergillus nidulans: effect of pentachloronitrobenzene (PCNB) and acenaphthene on the number of nuclei, number of hyphal tips and cell size of colonies of wild-type and PCNB-resistant strains 13 h after inoculation

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>PCNB</th>
<th>Acenaphthene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Resistant</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Colonies examined</td>
<td>10</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Hyphal length (µm)</td>
<td>1987·3</td>
<td>1834·0</td>
<td>119·0</td>
</tr>
<tr>
<td>Hyphal tips</td>
<td>19·4</td>
<td>10·7</td>
<td>6·4</td>
</tr>
<tr>
<td>No. of cells</td>
<td>11·5</td>
<td>9·3</td>
<td>6·7</td>
</tr>
<tr>
<td>No. of nuclei</td>
<td>222·6</td>
<td>139·0</td>
<td>26·2</td>
</tr>
<tr>
<td>Cell length (µm)</td>
<td>179·3</td>
<td>183·0</td>
<td>16·6</td>
</tr>
<tr>
<td>Nuclei/cell</td>
<td>20·1</td>
<td>14·9</td>
<td>4·0</td>
</tr>
</tbody>
</table>

Changes in the chemical composition of hyphal walls

In view of the striking cytological effects of PCNB it was thought worthwhile to find out if these were associated with changes in chemical composition. A PCNB-sensitive strain (bi-1; w-3; pyro-4) was grown in liquid CM and in CM containing 125 µg PCNB/ml. Wall samples were hydrolysed in 3N-HCl and in 8N-H2SO4 for 3·5 h at 105°C and at that temperature in 3N-HCl for 23·5 h in vacuo. Hydrochloric acid was removed in a vacuum desiccator over solid KOH. The sulphuric acid extract was neutralized with barium hydroxide and the precipitate removed by centrifugation. The precipitate was washed and the washings combined and made up to 100 ml with distilled water. The hexosamine content of the hydrolysates prepared by these methods did not differ significantly from one another; the method finally adopted is described under Methods. As a result of exposure to PCNB the hexosamine content of the wall was increased by one half.

The culture filtrate of PCNB-sensitive strains grown without but not with PCNB are very viscous. Eveleigh & Gorin (1969) have shown that Aspergillus nidulans produces an extracellular polysaccharide containing N-acetylgalactosamine and galactose units. A sample of the precipitated polysaccharide was hydrolysed and found to contain 39·5% by weight of hexosamine estimated as glucosamine.

The effect of PCNB, acenaphthene and sodium deoxycholate on growth, polysaccharide
Table 4. Effect of pentachloronitrobenzene (PCNB), acenaphthene and sodium deoxycholate on mycelium dry weight, extracellular polysaccharide and cell wall hexose and hexosamine content of wild-type and PCNB-resistant (penb-2) strains of Aspergillus nidulans

Cell wall and polysaccharide values the mean of three cultures; hexosamine and hexose value the mean of four estimations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Strain</th>
<th>Dry wt (mg) of cell walls/100 ml medium</th>
<th>Hexosamine (%)</th>
<th>Hexose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Wild-type</td>
<td>568</td>
<td>37</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>Resistant</td>
<td>636</td>
<td>39</td>
<td>18.7</td>
</tr>
<tr>
<td>PCNB</td>
<td>Wild-type</td>
<td>534</td>
<td>5</td>
<td>39.9</td>
</tr>
<tr>
<td></td>
<td>Resistant</td>
<td>503</td>
<td>39</td>
<td>24.4</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>Wild-type</td>
<td>634</td>
<td>45</td>
<td>23.1</td>
</tr>
<tr>
<td></td>
<td>Resistant</td>
<td>425</td>
<td>58</td>
<td>20.3</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>Resistant</td>
<td>464</td>
<td>8</td>
<td>21.3</td>
</tr>
</tbody>
</table>

Table 5. Aspergillus nidulans: Effect of age of culture and of pentachloronitrobenzene (PCNB) on extracellular polysaccharide production by wild-type and PCNB-resistant strains grown in liquid complete medium

Each value the average of two cultures.

<table>
<thead>
<tr>
<th>Age of culture (days)</th>
<th>Control</th>
<th>PCNB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Resistant</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>68</td>
<td>76</td>
</tr>
<tr>
<td>10</td>
<td>63</td>
<td>73</td>
</tr>
</tbody>
</table>

Table 6. Aspergillus nidulans: Percentage loss in weight on alkali extraction of wild-type and pentachloronitrobenzene (PCNB)-resistant walls of fungi grown with and without PCNB

Each value the mean of 12 estimations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PCNB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>42.3</td>
<td>34.5</td>
</tr>
<tr>
<td>PCNB-resistant</td>
<td>43.1</td>
<td>38.2</td>
</tr>
</tbody>
</table>

production and on the hexose and hexosamine content of hyphal walls of 7-day-old cultures of wild-type and resistant strains is shown in Table 4.

PCNB increased the hexosamine content of wild-type hyphal walls while the hexose content was lowered. The hexosamine content of the resistant strain was lower than that of the wild-type but was increased in the presence of PCNB; in both, the increase was about one-third. Deoxycholate appeared to have no significant effect on either the hexosamine or hexose content of either strain. Wild-type conidia failed to germinate in the presence of acenaphthene but those of the resistant strain did and grew. PCNB drastically lowered the yield of extracellular polysaccharide in the wild-type but had no effect on the resistant
Aspergillus nidulans: cell wall changes

Aspergillus nidulans mutant. Acenaphthene decreased the yield of polysaccharide in the resistant strain to about the same extent that PCNB did in the wild-type strain. Both strains synthesized similar amounts under normal conditions while deoxycholate increases polysaccharide production, especially by the resistant strain.

The polysaccharide might have been a product of lysis in staling cultures. Table 5 shows that polysaccharide was present 3 days after inoculation and reached a maximum 3 days later in the control and 7 days later in the resistant strain grown in the presence of PCNB. The polysaccharide would therefore appear to have been a normal product of fungal metabolism.

Changes in the hexosamine content of walls may embrace changes in the proportion of glucosamine and galactosamine. This was investigated by the method of Johnston (1963). However there was so much variation between samples that this work was discontinued.

PCNB may induce changes in other polysaccharide components of the wall. Table 6 shows the average loss in weight of samples of wall material after extraction in 2N-NaOH. Analysis of the data showed that PCNB has a highly significant effect in lowering the amount of alkali-extractable wall material in both strains. There was no difference between the strains grown without PCNB.

DISCUSSION

Several changes could be detected in the cytology and biochemistry of Aspergillus nidulans exposed to PCNB. Growth of hyphae was restricted so that they were about one-twentieth the length of those grown without inhibitor. The effect on the dry weight of mycelium grown in liquid medium was less marked. This may have been because of the different physical conditions and partly because of the thicker hyphal walls. PCNB decreased production of extracellular polysaccharide although deoxycholate increased it. Synthesis of the polymer itself may have been inhibited or there may have been inhibition of the synthesis of the galactosamine and/or galactose components. Changes in wall permeability which are known to be under genetic control in Neurospora crassa (Trevithick & Metzenberg, 1966) may have prevented secretion of the polymer so that it was deposited on the wall. That this might have been so was shown by removal of this thickening by amyloglucosidase which would have been able to hydrolyse the alpha linkages which predominate in the polymer (Gorin & Eveleigh, 1970) and by the observation that cells differed in their behaviour to tetrazolium and susceptibility to acid hydrolysis. The function of the polymer is unknown. In N. crassa galactosamine polymers in the wall bind polyphosphate and other anions (Harold, 1962). In A. parasiticus the galactosamine content of hyphal walls decreases with age (Distler & Roseman, 1960) while in A. niger it is highest in slow-growing cultures (Johnston, 1965). The osmotic mutant os-1 of N. crassa has galactosamine in the wall (Trevithick & Metzenberg, 1966) but it is low in mutant os-4. Glucosamine: galactosamine ratios may thus be important in determining wall properties and merit further investigation. In contrast to the behaviour of the wild-type the resistant strain made appreciable growth in the presence of PCNB, its hyphae appeared normal and it continued to secrete polysaccharide.

The alkali-soluble material of the wall decreased in the presence of PCNB, in the wild-type more than in the resistant strain. A decrease has been reported in os-1, os-3 and os-5 osmotic mutants of Neurospora crassa (Livingston, 1969). Sodium deoxycholate and acenaphthene had no effect on the hexosamine content of walls but PCNB was found to increase it by about one-third. This is in contrast to the results of Makris & Geogopoulos (1969) who found PCNB decreased the hexosamine content of walls of N. crassa. The
hexosamine content of the resistant strain was less than that of the wild-type but increased with PCNB to a value equal to that found in the wild-type grown without PCNB. A change in hexosamine content in *Aspergillus nidulans* was noted by Cohen, Katz & Rosenberger (1969) who found it to decrease in a temperature sensitive osmotic mutant.

Sorbose restricts growth of *Neurospora crassa* and increases the hexosamine content of its walls (de Terra & Tatum, 1961). It is thought to act at the hyphal apex by preventing intercalation of glucose units (Rizvi & Robertson, 1965) so that the amount of wall glucan is decreased. Pentachlorophenol acts in a similar way to sorbose (Crocken & Tatum, 1968). Chlorinated nitrobenzenes may also act at the cell surface, and the observation that less tetrachloronitrobenzene could be extracted from a resistant mutant than from a sensitive (Threlfall, 1968) could partly explain resistance.

These results demonstrate the pleiotropism of the resistant strain so that, although the primary biochemical lesion has not been discovered, changes in the synthesis of wall components are an inherited feature associated with resistance although not necessarily a cause of it.

**REFERENCES**


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