The Genetics and Biochemistry of Mutants of *Aspergillus nidulans* Resistant to Chlorinated Nitrobenzenes

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SUMMARY

2,3,5,6-Tetrachloronitrobenzene (TCNB) was a more effective fungistat than pentachloronitrobenzene (PCNB) for *Aspergillus nidulans*. Four mutants selected for resistance to TCNB or PCNB were resistant to both these compounds and to other halogenated nitrobenzenes, diphenyl, methylene blue and brilliant cresyl green. Resistance was conferred by either of two recessive genes in linkage group III. Three of the mutants were allelic. In crosses the frequency of resistant ascospores was less than 50%. Diploids heterozygous for resistance exposed to PCNB produced fast-growing resistant haploid or homozygous diploid segregants. PCNB decreased growth more than DNA synthesis in sensitive strains but these were unaffected in resistant strains. Five times as much TCNB was extracted from the mycelium of sensitive strains than from that of resistant strains. Resistance is probably caused by an inability to take up the chemicals rather than to an ability to metabolize them.

INTRODUCTION

When a chemical compound is used to control or prevent the growth of a micro-organism strains resistant to the compound may subsequently appear. How the compound acts on the organism and how this action is avoided or overcome by the resistant strain has considerable biochemical and genetical interest. Pentachloronitrobenzene (PCNB) and 2,3,5,6-tetrachloronitrobenzene (TCNB) are widely used for the control of diseases caused by *Rhizoctonia solani* and *Botrytis cinerea* (Brown & Montgomery, 1948). Strains of the imperfect fungi *B. allii* (Priest & Wood, 1961) and *Fusarium caeruleum* (McKee, 1951) resistant to chlorinated nitrobenzenes have been described, but not analysed genetically. In *Hypomyces solani* f. *cucurbitae* any one of three loci can mutate to give resistance to PCNB and TCNB (Georgopoulos, 1963). In *Aspergillus nidulans*, strains resistant to acriflavine (Roper & Käfer, 1957), fluoroacetate (Apirion, 1962) and *p*-fluorophenylalanine, teoquil, iodoacetate, actidione and malachite green (Warr & Roper, 1965) have been described and analysed genetically. No reports have appeared on strains of *A. nidulans* resistant to fungicides used in agriculture. This is perhaps not surprising, because the fungus is not a pathogen. The ease with which genetic analysis can be done with *A. nidulans* was the reason for its use in this study of the nature of its resistance to PCNB and TCNB.

METHODS

The general and genetic techniques used in this work were those of Pontecorvo *et al.* (1953). Incubation was at 37°.

Organisms. Mutant stocks of *Aspergillus nidulans* were obtained from Dr B. W. Bainbridge (Department of Microbiology, Queen Elizabeth College, Campden Hill Road, London, W.8), the Department of Genetics University of Glasgow, or the Fungal Genetics Stock Center, Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire, U.S.A. The mutants used in this work were *y*, yellow conidia; *w2*, white conidia; *ad20*, *arg2*, *bi1*, *lys5*, *meth2*, *nic8*, *phen2*, *pro1*, *pyro4*, *ribo2*, *s3* growth requirements, respectively, for adenine, arginine, biotin, lysine, methionine, nicotinic acid, phenylalanine, proline, pyridoxine, riboflavin and sulphite; *gal1*, unable to grow with galactose as sole carbon source; *Acr1*, resistance to acriflavine and *su1 ad20*, suppressor of *ad20*. Details of these mutant alleles are given in Pontecorvo *et al.* (1953), Roper & Käfer (1957), Käfer (1958) and Roberts (1963). These stocks were maintained on complete medium. Where necessary these stocks and others obtained during the work were purified by the isolation of a single conidium with a de Fonbrune micromanipulator.

Media. Minimal medium (MM): NaNO_3 , 6.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52 g.; KCl, 0.52 g.; KH_2PO_4 , 1.52 g.; glucose, 10.0 g.; trace FeSO_4 and ZnSO_4 ; distilled water to 1 l.; adjusted to pH 6.5 with 5% (w/v) NaOH. Complete medium (CM): yeast extract (Difco), 1.0 g.; peptone (Difco), 1.0 g.; Casamino acids (Difco, certified), 1.0 g.; glucose, 10.0 g.; vitamin solution, 1 ml. (biotin, 0.01 g.; thiamine HCl, 0.01 g.; riboflavin, 0.01 g.; *p*-aminobenzoic acid, 0.01 g.; nicotinic acid, 0.01 g.; pyridoxin HCl, 0.01 g.; water to 100 ml.); distilled water to 1 l., pH 6.0. For the growth of adenine auxotrophs media were supplemented with 0.15 g. adenine/l. CM was deficient in arginine, methionine, phenylalanine and riboflavin so that these were added to the media when required by mutant stocks. Media were solidified by the addition of 15 g. Oxoid Ionagar no. 2/l. Cultures grown in liquid media were set up in 250 ml. Oxoid flasks containing 50 ml. medium. The components of liquid MM were autoclaved separately, mixed, the pH adjusted with 5% (w/v) NaOH and dispensed into sterile flasks. Liquid CM was prepared in bulk, distributed and sterilized in the flasks.

Chemicals. The five fungicides used were pentachloronitrobenzene (PCNB), 2,3,5,6-tetrachloronitrobenzene (TCNB) and 2,5-dibromonitrobenzene (DBNB) recrystallized from ethanol and 1,3-difluoro-7,6-dinitrobenzene (DFDNB) recrystallized from acetic acid. Other chemicals were of Analar grade where available. Stains were obtained from G. T. Gurr (136/177, New Kings Road, London, S.W.6). Purified calf thymus DNA, glucose oxidase and peroxidase were purchased from Seravac Laboratories, Maidenhead, Berkshire.

Analysis of cultures grown in liquid media. The fungal mycelium was collected by filtration through Whatman no. 571 filter paper and when necessary a sample of the filtrate was stored at -20° for analysis later. The mycelium was washed with water, twice with 25 ml. industrial methylated spirit (74 o.p.), then with 25 ml. acetone, squeezed between blotting paper, removed from the paper, dried on a glazed tile at $55-60^\circ$ for at least 4 hr and then weighed. DNA was measured as follows. Not more than 100 mg. dry mycelium was extracted twice with 2.5 ml. and then with 5.0 ml. ethanol+chloroform mixture (3+1, v/v) for periods of 20 min. at 75° . It was then extracted with 2.5 ml. 6% (v/v) perchloric acid for 20 min. at 70° . To 2.0 ml. of the perchloric acid extract was added 4.0 ml. diphenylamine reagent (Burton, 1956), incubated overnight at 37° and the extinction read at 600 m μ . A sample of calf thymus

DNA was hydrolysed and standards prepared at the same time. Culture filtrates were analysed for phosphate by the method of Fiske & SubbaRow (1925), for glucose with a glucose oxidase reagent in 0.5 M-tris at pH 7.0 (Dahlqvist, 1961) and for amino acids by the method of Moore & Stein (1948) with leucine as standard.

Analysis of 2,3,5,6-tetrachloronitrobenzene. A modification of the method of Auerbach (1950) was used. One-half ml. of a 25 % (v/v) solution of tetra-ethylammonium hydroxide (British Drugs Houses Ltd., Poole, Dorset) was diluted with 12.5 ml. absolute ethanol. To 0.1 ml. of this was added 4.9 ml. acetone extract containing 0.60 µg. TCNB and the extinction read at 550 mµ between 4 and 7 min. after mixing.

Cytological technique. Strips of thin sterile Cellophane (2 × 1 in.) were placed on the surface of CM agar in Petri dishes and inoculated with a suspension of conidia. After incubation the strips were removed, fixed in Helly's fluid for 10 min., washed in 70 % (v/v) ethanol in water, hydrolysed with N-hydrochloric acid at 60° for 10 min. and stained in diluted Giesma for 3 hr (Giesma R 66 1.5 ml.; M/15 phosphate buffer, pH 6.9, 30 ml.). The material was differentiated by washing the strips for about 75 sec. in distilled water containing a trace of acetic acid. The strips were mounted for examination in dilute stain (Giesma R 66, 7 drops; M/15 phosphate buffer, pH 6.9, 20 ml.).

RESULTS

Effect of PCNB and TCNB on growth and morphology of wild type

A series of Petri dishes (8.5 cm. diameter) of CM agar containing known amounts of PCNB (0–0.5 mg./ml.) were prepared by adding PCNB dissolved in acetone to the warm medium before the plates were poured. The plates were inoculated with about 200 conidia/plate. After incubation for 3 days the colonies were counted. On plates containing more than 0.05 mg. PCNB/ml. the colonies did not exceed 2 mm. in diameter. There was no significant difference ($P = 0.01$) between the control and PCNB plates. A similar experiment was made with 0.01–10.0 mg. PCNB deposited in the lids of a series of dishes from acetone solutions. Again the viability of conidia was unchanged.

The effect of PCNB on growth was examined by inoculating a series of plates at the centre with a 2 mm. disc of mycelium cut with a cork borer from the periphery of a colony growing on CM agar. The average diameter of the colonies after 6 days is shown in Fig. 1. At 0.005 mg. PCNB/ml. growth was decreased, while at 0.05 mg./ml. the colonies were only about one-sixteenth the area of control colonies. Increasing the concentration above 0.05 mg./ml. did not lead to greater inhibition. At concentrations above 0.01 mg./ml. conidiation was progressively decreased and was almost absent at 0.05 mg./ml.

TCNB also decreased growth and conidiation; above 0.05 mg./ml. there was no conidiation and the colonies were bright pink. At 0.1 mg./ml. inhibition was complete but the organisms were not dead because growth started again on transfer of the inoculum to medium without TCNB. Thus PCNB and TCNB were fungistatic rather than fungicidal.

Isolation of PCNB- and TCNB-resistant strains

At any time during exposure to PCNB fan-shaped fast-growing sectors developed. Ten sectors taken at random, each from different colonies of the wild type growing on CM agar and exposed to the vapour from 1.0 mg. PCNB in the lid of the dish,

were examined in detail. They were found to differ from each other in several ways. Three of the sectors grew poorly on CM agar, produced an excess of aerial hyphae with few conidiophores and the edge of the colony was indented. Looked at through the bottom of the dish these strains were dark brown. When grown on CM agar containing 0.2 mg. PCNB/ml. these strains were normal in every way. Three other sectors had normal morphology in the absence of PCNB but produced excessive aerial hyphae in its presence. Another three sectors appeared to be normal whether PCNB was present or not; one of these sectors had a growth rate similar to that of the wild-type parent but the other two grew slowly. The tenth sector lost its resistance to PCNB on subculture while the others retained their resistance through several transfers by using conidia or mycelium. None of the resistant strains derived from the wild-type parent had any nutritional requirement.

Table 1. *Strains of Aspergillus nidulans resistant to pentachloronitrobenzene (PCNB) and tetrachloronitrobenzene (TCNB)*

Genotype	Gene for resistance	Isolated in presence of
<i>pro 1 y; Acr 1</i>	<i>pcnb 1</i>	PCNB
<i>ad 20; pyro 4</i>	<i>pcnb 2</i>	PCNB
<i>pro 1 y; Acr 1</i>	<i>pcnb 3</i>	PCNB
<i>bi 1</i>	<i>tcnb 1</i>	TCNB
<i>pro 1 y; Acr 1; pcnb 3</i>	<i>pcnb 3 A</i>	Dibromonitrobenzene

Plates of CM agar containing 0.2 mg. PCNB/ml. were inoculated with mutant auxotrophic strains of *Aspergillus nidulans*. After incubation one resistant sector was picked. Repeated attempts to isolate a resistant strain in the presence of TCNB were unsuccessful. This may have been because so little growth was made that the probability of a mutation to resistance taking place among so few nuclei was slight. Therefore plates of CM agar were inoculated and 10 mg. TCNB added to the lid after 2 days. After further incubation a few tolerant sectors developed but their frequency was much less than that of sectors developing in the presence of PCNB. One sector resistant to TCNB and three resistant to PCNB have been studied. The genotypes of the parental strains are shown in Table 1, where resistance has been tentatively ascribed to four genes. On CM agar in the presence of dibromononitrobenzene the original *pro 1 y; Acr 1; pcnb 3* strain produced a fast-growing sector which still carried *pcnb 3*. The strain grown from this sector was called *pcnb 3 A*; a second mutation may have occurred, either at the *pcnb 3* locus or elsewhere, but this was not investigated.

Response of resistant strains to other inhibitors

The effect of several chemicals on the growth of the isolates of Table 1 and of prototrophic-resistant recombinants obtained from crosses (see Table 2) was measured by the average of two diameters at right angles of colonies incubated for 6 days on CM agar containing the chemical. The source of inoculum, whether taken from cultures grown with or without PCNB (or TCNB) had no effect on the final size of the colony. Figure 1 shows that the strain having the greatest resistance to PCNB was that isolated in the presence of TCNB. In the absence of PCNB strains *pcnb 1* and *pcnb 2* grew much less than the other strains; *pcnb 2* was stimulated by PCNB. At high

concentrations of PCNB growth of *pcnb 1* was decreased more than that of *pcnb 2* or *pcnb 3*. These properties were shown on MM agar but the differences between strains was less marked. Figure 2 shows that the strain selected in the presence of TCNB was no more resistant to it than were those selected in the presence of PCNB. At more than 0.05 mg. TCNB/ml. growth of all resistant strains was suppressed and all became pink in colour. Strains carrying *pcnb 1* or *pcnb 2* were found to be resistant to dichloronitrobenzene and dibromonitrobenzene at concentrations up to 0.1 mg./ml. All strains were completely inhibited by difluorodinitrobenzene at 0.01 mg./ml. and by dinitrophenol at 0.05 mg./ml. Priest & Wood (1961) found that strains of *Botrytis*

Fig. 1. *Aspergillus nidulans*: diameters of colonies of wild type (sensitive) and *pcnb 1*, *pcnb 2*, *pcnb 3* and *tcnb 1; bi 1* (resistant) after incubation for 6 days at 37° on complete medium containing 0–500 μg. pentachloronitrobenzene (PCNB)/ml. (means of four replicates). ●, Wild type; —○—, *pcnb 1*; —○—, *pcnb 2*;○....., *pcnb 3*; —■—, *tcnb 1; bi 1*.
Fig. 2. *Aspergillus nidulans*: diameters of colonies of wild type (sensitive) and *pcnb 1*, *pcnb 2*, *pcnb 3* and *tcnb 1; bi 1* (resistant) after incubation at 37° for 6 days on complete medium containing 0–500 μg. 2,3,5,6-tetrachloronitrobenzene (TCNB)/ml. (means of four replicates). ●, Wild type; —○—, *pcnb 1*; —○—, *pcnb 2*;○....., *pcnb 3*; —■—, *tcnb 1; bi 1*.

allii resistant to chloronitrobenzenes were also resistant to 2,6-dichloronitroaniline (DCNA). *Aspergillus nidulans* was insensitive to DCNA; no difference in sensitivity of sensitive and chloronitrobenzene-resistant strains was shown at 0.25 mg. DCNA/ml. At this concentration the medium was bright yellow, but surrounding each colony there was a bleached border, suggesting that the compound may have been destroyed. Georgopoulos & Vomvouianni (1965) noted that mutants of *Hypomyces solani* f. *cucurbitae* resistant to PCNB and TCNB were also resistant to diphenyl. With *A. nidulans* all the chloronitrobenzene-resistant mutants were able to grow on media containing 0.05 mg. diphenyl/ml. Under these conditions the wild type sensitive strain could not grow. Conidiation of strains *pcnb 1* and *pcnb 3* was unchanged but that of *pcnb 2* and *tcnb 1* was diminished. All the resistant strains were able to grow and conidiate normally on CM agar containing 0.05 mg. methylene blue or brilliant cresyl/ml., whereas the wild-type strain grew poorly and did not produce conidia.

Genetics of chloronitrobenzene resistance

A prototrophic diploid of *Aspergillus nidulans* was selected from a stable heterokaryon between *pro 1 y; Acr 1; pcnb 1* and *su 1 ad20 yad20; Acr 1; phen 2; pyro 4; lys 5; s 3; nic 8; ribo 2*. Loopfuls of diploid conidia were stabbed into plates of CM agar at five equidistant points and 1.0 mg. PCNB added to the lid. After incubation for 3 days, fast-growing sectors appeared from the stunted colonies. The sectors were either dull yellow (diploid) or bright yellow (haploid) and about equally frequent. One sector was picked from each colony and analysed genetically. All the haploids were

Table 2. *Aspergillus nidulans*: crosses between chloronitrobenzene-resistant strains

Cross no.	Parental genotypes	Selection	No. of ascospores analysed	No. resistant
1	$\frac{+ \text{ ad20 } + \text{ pcnb 2 } + \text{ pyro 4 }}{y + \text{ Acr 1 pcnb 1 arg 2 gal 1 } +}$	<i>ad20⁺ arg 2⁺ pyro 4⁺</i>	900 (897y; 3y ⁺)	900
2	$\frac{y \text{ pro 1 pcnb 1 } + \text{ nic 8 } +}{y \text{ pro 1 pcnb 3 pyro 4 } + \text{ s 3}}$	<i>pyro 4⁺ nic 8⁺ s 3⁺</i>	821	821
3	$\frac{y + \text{ pcnb 3 pyro 4 s 3 }}{+ \text{ ad20 pcnb 2 } + \text{ +}}$	<i>ad20⁺ pyro 4⁺</i>	251 (212y; 39y ⁺)	251
4	$\frac{+ \text{ bi 1 } + \text{ pcnb 2 arg 2 } +}{y + \text{ meth 2 tcnb 1 } + \text{ phen 2}}$	None	98	97

Table 3. *Aspergillus nidulans*: crosses of chloronitrobenzene strains to *bi 1 arg 2*

Cross no.	Parental genotypes	Meiotic products				Recombination (%)
		<i>pcnb arg 2</i>	<i>+</i>	<i>+</i>	<i>pcnb + + arg 2</i>	
5	$\frac{\text{pro 1 } y + \text{ Acr 1 pcnb 1 } +}{+ + \text{ bi 1 } + \text{ + arg 2}}$	11	40	130	223	12.6
6	$\frac{\text{ad20 } + \text{ pcnb 2 } + \text{ pyro 4 }}{+ \text{ bi 1 } + \text{ + arg 2 } +}$	12	24	49	97	19.7
7	$\frac{\text{pro 1 } y + \text{ Acr 1 pcnb 3 } + \text{ pyro 4 s 3 }}{+ + \text{ bi 1 } + \text{ + arg 2 } + \text{ +}}$	12	17	43	71	20.3
8	$\frac{\text{pro 1 } y + \text{ Acr 1 pcnb 3 A } +}{+ + \text{ bi 1 } + \text{ + arg 2}}$	39	8	88	212	13.6

phen⁺ but showed assortment of the other heterozygous markers. Thus *pcnb 1* is located in linkage group III; *pcnb 3* and *tcnb 1* were also located on this chromosome by the same method. Attempts to measure the growth rate of diploids heterozygous for resistance in the presence of PCNB failed because fast-growing sectors appeared sooner or later. However the sensitivity of heterozygous diploids indicated that resistance behaved as a recessive character. Table 2 shows that in crosses between resistant mutants *pcnb 1*, *pcnb 2* and *pcnb 3* there were no sensitive recombinants among the several hundred analysed; this suggests that the three genes are either closely linked or alleles. In a cross between *pcnb 2* and *tcnb 1* (cross no. 4) one sensitive recombinant *y* was found in 98 analysed, so these genes may be distinct. Table 3

shows that the *pcnb* locus is linked to *arg 2* but the frequency of resistant ascospores is less than half so that the marker is unsuitable for use in meiotic analysis. A three-point cross was made between *bi 1;tcnb 1* and *y;meth 2 gal 1;nic 8;ribo 2*. Of 108 ascospores analysed 79 (15+64) were non-crossovers. There were 11 (6+5) crossovers between *meth 2* and *tcnb 1*, 17 (3+14) between *tcnb 1* and *arg 2*, and 1 (1+0) with crossing-over in both regions. (Figures in parentheses refer to number of resistant and sensitive ascospores respectively.) The results indicate that *tcnb 1* is situated between *meth 2* and *gal 1*. A diploid heterozygous for *pcnb 2* and *arg 2* in coupling was exposed to PCNB and of 18 resistant diploid recombinants all required arginine, suggesting that they are homozygous for *arg 2* and indicating that the gene for resistance is between *arg 2* and the centromere. Slow-growing resistant strains occasionally gave faster-growing sectors which retained the resistance of the parent. The possibility that this was due to mutations taking place elsewhere in the resistance cistron or elsewhere in the genome has not been explored.

The mechanism of chloronitrobenzene action and the nature of resistance

The chloronitrobenzenes are almost insoluble in water (PCNB 1.5 μ mole/l.) but may accumulate in the biophase and act as toxicants in a non-specific way. Any change which decreased uptake might confer some degree of tolerance on the fungus. That there were differences of this sort was shown by the following experiment. A sensitive (wild type) and resistant (*pcnb 1*) strain of *Aspergillus nidulans* were grown in liquid CM for 3 days and then 10 mg. TCNB added to each flask. After incubation for a further 3 days the mycelium was collected, washed, dried, weighed and extracted for 24 hr with acetone at 4°. The TCNB in the extracts was then estimated. Table 4 shows that more than five times as much TCNB was extracted from the sensitive mycelium as from the resistant mycelium.

Cytological examination of hyphae produced by conidia incubated overnight in an atmosphere of PCNB showed striking differences between sensitive and resistant strains. Sensitive strains developed short much-branched hyphae with many cross-walls. The cells had thick walls and were crowded with nuclei. Under the same conditions conidia of resistant mutants gave long thin hyphae with few cross-walls and few nuclei per cell, and were indistinguishable from hyphae of either strain grown in the absence of PCNB. These effects were studied quantitatively on cultures grown in liquid MM containing 0.1 mg. PCNB/ml. Table 5 shows that PCNB depressed growth more than DNA synthesis in the sensitive strain, so that the DNA content was increased. There was no significant effect of PCNB on the resistant strain. Even in the absence of the fungistat the sensitive strain utilized glucose at a rate lower than did the resistant strain. There were no significant differences between the inorganic phosphate content of culture filtrates. When this experiment was repeated with 0.1 mg. TCNB/ml. the results shown in Table 6 were obtained. No values for the sensitive strain with TCNB are given because no growth was made. These experiments show that PCNB did not inhibit mitosis as suggested by Horsfall (1956) although TCNB may do so.

PCNB and TCNB may release nascent chlorine which could act as an oxidizing agent and/or produce an antimetabolite by causing chlorination of a metabolite (Rich, 1960). Chloramine T is known to act in this way but there was no difference between sensitive and resistant strains in their response to this chemical. Rich (1960)

also suggested that chloronitrobenzenes might be competitive inhibitors of inositol synthesis. Addition of 0.05–0.50 mg. inositol/ml. had no effect on the growth of sensitive strains in the presence of PCNB.

Table 4. *Aspergillus nidulans*: tetrachloronitrobenzene content of resistant and sensitive strains

Strain (genotype)	Dry wt. mycelium (mg.)	$\mu\text{g. TCNB/mg. mycelium}$
Sensitive (wild type)	98.9	35.4
Resistant (<i>pcnb</i> 1)	268.5	6.5

Each value is the mean of five replicate cultures.

Table 5. *Aspergillus nidulans*: effect of 0.1 mg. PCNB/ml. on mycelium dry weight, DNA content and glucose uptake of sensitive and resistant strains

Strain	Treatment	Mycelium dry wt. (mg.)	Total DNA ($\mu\text{g.}$)	DNA ($\mu\text{g./100 mg. dry mycelium}$)	Glucose ($\mu\text{g./ml. culture filtrate}$)
Sensitive (wild type)	PCNB	69	247	362	6800
	Control	151	324	216	2740
Resistant (<i>pcnb</i> 3)	PCNB	145	336	232	17
	Control	162	324	230	123

Each figure is the mean of five replicate cultures.

Table 6. *Aspergillus nidulans*: Effect of 0.1 mg. TCNB/ml. on mycelium dry weight, DNA content and glucose uptake of sensitive and resistant strains

Strain	Treatment	Mycelium dry wt. (mg.)	Total DNA ($\mu\text{g.}$)	DNA ($\mu\text{g./100 mg. dry mycelium}$)	Glucose ($\mu\text{g./ml. culture filtrate}$)
Sensitive (wild type)	Control	119	701	477	3090
Resistant (<i>pcnb</i> 3)	TCNB	18	232	1329	10400
	Control	136	698	515	520

Each figure is the mean of five cultures.

If PCNB or TCNB act as uncouplers of oxidative phosphorylation respiration would be stimulated, and the loss of energy by failure of ATP synthesis might be enough to retard growth. No change in the respiration rate of sensitive or resistant strains was detected over a period of 2 hr after the addition of 0.125 mg. PCNB or TCNB/ml.

PCNB and TCNB might increase cell permeability so that loss of low molecular weight substances limited growth, but no differences in the amino acid content of culture filtrates from sensitive or resistant cultures was detected 1 or 6 days after the addition of PCNB to cultures to give a concentration of 0.4 mg./ml.

DISCUSSION

Resistant mutants of *Aspergillus nidulans*, which appear to arise more frequently in the presence of PCNB than with TCNB, are mutant for a gene in linkage group III. One gene may be responsible or two closely linked genes. Those resistant mutants selected by PCNB are allelic but have different growth rates and sensitivities to other chemicals. The production of homozygous resistant diploid segregants by sensitive heterozygous diploids to PCNB should provide a method for mapping genes on this arm of the chromosome by mitotic recombination. The cause of toxicity has not been discovered; inhibition of inositol metabolism or evolution of nascent chlorine appear unlikely to explain the fungistatic action of PCNB or TCNB.

Lehninger (1949) showed that when methylene blue or brilliant cresyl green substitutes for the cytochrome system in electron transport there is an uncoupling of ATP synthesis. The resistance to the dyes shown by PCNB- and TCNB-resistant mutants may indicate that these compounds also act in this fashion. However, they may act quite differently inside the cell but share the same path for uptake or entry. If this path is blocked in a mutant strain then resistance will be shown to all those compounds that use it. This line of argument can account for resistance to other halogenated nitrobenzenes and to diphenyl.

The high content of TCNB extracted from a sensitive strain of *Aspergillus nidulans* as compared with a resistant strain may reflect either the inability of the resistant strain to accumulate TCNB or its ability to inactivate it. The fate of halogenated nitrobenzenes *in vivo* has been studied in the rat and rabbit but not in fungi. The labile nitro group of PCNB and TCNB is replaced by a cysteinyl group with the formation of a mercapturic acid (Bray, Hybs, James & Thorpe, 1953); cysteine required for this synthesis is derived from glutathione by a specific enzyme glutathione S-transferase (Al-Kassab, Boyland & Williams, 1962; Booth, Boyland & Sims, 1961). Georgopoulos & Vomvouianni (1965) looked for this enzyme in PCNB-resistant mutants of *Hypomyces solani* f. *cucurbitae* without success. They pointed out that if it had been present it could not have accounted for resistance to diphenyl. Resistance would therefore seem to result from a decreased ability to accumulate the toxic chemical rather than from an ability to metabolize it to some harmless product. *A. nidulans* is more sensitive to TCNB than PCNB; *Botrytis cinerea* behaves in this way (Reavill, 1954), whereas the reverse is true for *Rhizoctania solani* (Brown & Montgomery, 1948). Both compounds are virtually inactive against *Pythium* and *Phytophthora* (Reavill, 1954). These variations in sensitivity may indicate differences in the nature or organization of the hyphal wall.

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