Induced Sectoring in Diploid Aspergillus nidulans as a Criterion of Fungitoxicity by Interference with Hereditary Processes

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ABSTRACT

A green diploid strain of *Aspergillus nidulans*, heterozygous for the recessive genes determining production of yellow and white conidia, was used to study the ability of fungicides to induce somatic segregation. Comparison of eight antifungal compounds of known, site-specific, inhibitory action showed that induced sectoring is specific for compounds acting on hereditary processes. Among nine other agricultural fungicides with either a multisite, or an uncertain, mechanism of action, several members of the aromatic hydrocarbon group were shown to possess genetic activity. Although these fungicides may have other effects on cellular activities, it is believed that a site within the cell nucleus is probably the most sensitive one.

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Interference with some of the processes within the cell nucleus has long been recognized as a possible mechanism of fungicidal action (15). Inside the nucleus, a fungicide may interfere with nucleic acid synthesis; chromosome division; mitotic spindle formation, orientation, or function; induce gene mutations or chromosome aberrations; or increase recombination frequency. Since not all of these effects can be recognized by cytological examination, genetic tests often can give valuable information. For example, if a compound which prevents the separation of daughter chromosomes at mitosis were tested at concentrations which permit some growth, it would be logical to expect that it would have no effect on normal division of some nuclei, completely prevent it in others, and cause nondisjunction of occasional chromosome pairs in still others. The latter effect would induce formation of aneuploid nuclei and, eventually, easily recognizable haploid and nondisjunctional diploid sectors (somatic segregants) in colonies of a diploid fungal strain, heterozygous for appropriate gene pairs (25). On the basis of such evidence, Hastie (13) suspected that benomyl may possess antimitotic activity 3 years before the mechanism of antifungal action of the benzimidazole-configuration fungicides was clarified by cytological and biochemical studies (5, 12).

Nondisjunction of chromosomes is not, of course, the only mechanism by which a diploid colony may produce sectors. Increased sectoring may also be caused by compounds that induce gene mutations or mitotic crossing over, leading to homozygosis for particular gene pairs, chromosome breakage and deletion, that result in hemizygosis or chromosome loss which may eventually cause haploidization. In each case recognition of the mechanism of induced sectoring may be possible if an appropriately marked diploid is used, and the genotypes of segregants are studied. In the present work, a number of commercial fungicides and some antifungal compounds of known mechanism of toxic action were used to show that the induced sectoring test is reliable for a first recognition of compounds the toxicity of which is due, at least in part, to their effect on nuclear function.

MATERIALS AND METHODS.—A prototrophic diploid strain of *Aspergillus nidulans* (Eidam) Winter was used for the detection of ability of fungicides to induce mitotic segregation. This strain produces green conidia, but carries the recessive genes for yellow *(y)* and white *(w)* conidia in the heterozygous condition so that upon mitotic segregation yellow and white sectors are produced (17). Conidia were plated on Pontecorvo's (21) minimal medium and incubated overnight. From such plates, blocks of inoculum (approximately 1 mm^2) were transferred to plates of complete medium (so that growth of segregants with nutritional requirements would not be prevented) with or without fungicide. Five blocks were transferred to each 9-cm diameter plate and were equidistantly arranged so each colony had equal space to grow and potentially equal opportunity to segregate. The number of yellow and white sectors on such colonies was recorded by macroscopic examination, following 7 days of incubation at 38 C. The ratio between the number of sectors in 100 treated and untreated colonies in each experiment was used as a criterion for the ability of a particular treatment to induce somatic segregation.

The antifungal compounds tested were obtained from commercial sources and from various colleagues. If not available in practically pure form, the chemical was recrystalized from an appropriate solvent. For compounds not sufficiently soluble in water, stock solutions in organic solvents were prepared and the amount required was added to partially cooled agar before plates were poured. The solvent concentration never exceeded 2% (v:v) in the agar medium and was found to be without effect on either growth or sectoring in control plates. In the case of nystatin and zineb, no solvent which might be used at nontoxic concentrations could be found. These chemicals were added directly to the melted agar. On the basis of preliminary toxicity tests, three concentrations of each fungicide were chosen to

produce different levels of inhibition of colony growth. For each of these concentrations approximately 100, but more than 80, colonies of the diploid *A. nidulans* were tested. Difficulty was encountered with zineb which, at the concentrations chosen, interfered with the normal appearance of the green color in the heterozygous diploid colonies. This effect seems to be due to chelation of some microelement important for pigmentation, for when the amount of the trace elements in the medium (21) was increased, pigmentation was restored. As expected, with higher concentrations of trace elements toxicity was reduced, but preliminary tests permitted selection of a medium composition at which the toxicity of zineb was not significantly affected, but its effect on colony color was eliminated.

RESULTS.—It is obvious from the data in Table 1 that not all compounds which inhibit growth affect the stability of the heterozygous diploid strain. The suitability of the induced sectoring test for the recognition of those chemicals which inhibit fungal growth by interference with some nuclear process is best illustrated by the study of compounds 1-8 in Table 1. For each of these compounds a specific biochemical mechanism of action is known. The frequency of mitotic segregation is completely unaffected by inhibitors of processes unrelated to the hereditary function. This category includes polyoxin D which acts as a competitive inhibitor for uridine diphosphate-N-acetylglucosamine in the chitin synthetase reaction (7), cycloheximide (actidione) which inhibits the transfer of amino acids from aminoacyl-tRNAs into peptide chains during cytoplasmic protein synthesis (26), carboxin which blocks mitochondrial electron transport at the level of succinic dehydrogenase (29), triarimol which is inhibitor of ergosterol biosynthesis (22), and nystatin which alters the permeability properties of the cell membrane (18). Even at highly inhibitory concentrations, these fungitoxicants had no effect on sectoring. In sharp contrast, all three site-specific fungicides used, which are known to act within the cell nucleus increase somatic segregation even at concentrations which cause only slight inhibition of growth (Table 1). Actinomycin D, an intercallator, which is known to bind to DNA (24), griseofulvin which causes a breakdown of mitotic spindles (4,11), and benomyl which probably has a similar action (5,12) were very effective inducers of sectoring in colonies of heterozygous diploid *A. nidulans*.

With the above knowledge, the effect of compounds 9-18 whose fungitoxicity has not been ascribed to a specific-site inhibition was examined. The dithiocarbamates, which include zineb, inhibit many different enzymes, presumably by forming heavy metal complexes (20). Reaction with thiols may be responsible for the toxicity of compounds similar in structure to daconil (3). Dodine is a surface-active agent which attacks membranes and causes leakage of metabolites (1). Dimethirimol presumably acts as an antagonist to pyridoxal which is involved through C-1 metabolism in the synthesis of important metabolites (2). These four fungicides, together with the recently introduced plondrel (the mechanism of action of which seems to be unknown) apparently do not interfere with hereditary processes since they have no effect on sectoring (Table 1). On the other hand, the results obtained with the fungicides PCNB, TCNB, dicloran, sodium orthophenyl phenate, and chloroneb are very striking. These fungicides are as effective inducers of sectoring as benomyl and griseofulvin (Table 1), although their ability

TABLE 1. Effect of fungicides on induced sectoring of heterozygous diploid colonies of *Aspergillus nidulans*

Compound	Concentration (μM)	Inhibition of growth[a] (%)	Induced segregation index[b]
1. Polyoxin D	2	12	1.11
	6	20	0.99
	9	40	1.03
2. Cycloheximide	70	27	1.04
	180	33	0.76
	540	63	1.28
3. Carboxin	2	18	0.70
	4	47	1.12
	12	77	0.92
4. Triarimol	3	32	1.03
	10	46	1.06
	30	60	1.00
5. Nystatin	10	7	0.97
	30	33	1.04
	60	48	0.95
6. Actinomycin D	8	7	2.89
	16	35	4.91
	24	60	6.92
7. Griseofulvin	17	15	3.84
	54	26	4.08
	108	52	4.48
8. Benomyl	0.75	23	3.89
	1.25	42	5.50
	1.75	61	6.39
9. Zineb	350	18	1.01
	1,000	29	1.06
	1,800	42	1.01
10. Daconil	1	20	1.06
	2	60	0.95
	3	90	1.01
11. Dodine	2.5	25	0.95
	5.0	32	1.00
	7.0	58	1.08
12. Dimethirimol	950	9	0.87
	1,900	32	0.73
	4,800	49	0.83
13. Plondrel	1,500	25	0.81
	2,000	32	1.06
	2,500	52	0.86
14. PCNB	5	13	3.38
	10	39	5.79
	17	70	7.11
15. TCNB	12	30	2.18
	18	54	2.36
	24	61	3.82
16. Dicloran	14	28	4.78
	24	60	5.87
	38	66	6.91
17. SOPP	16	30	2.09
	26	48	2.79
	52	69	4.65
18. Chloroneb	24	36	2.58
	34	48	3.49
	48	67	4.88

[a]Measured as percentage reduction of colony diameter following 5 days of incubation at 38 C.

[b]Induced segregation index = $\frac{\text{Number of sectors per 100 treated colonies}}{\text{Number of sectors per 100 control colonies}}$

to interfere with hereditary processes in fungi is not generally recognized. On the basis of cross resistance experiments these fungicides have been classified together in the aromatic hydrocarbon group (10,28). Members of the group have been suspected of having a number of effects on fungal cells, including inhibition of protein synthesis (30) and changes in membrane permeability (9) and wall composition (19,27). The ability of all of the aromatic hydrocarbon fungicides tested to increase the frequency of sectoring in the diploid *A. nidulans* is a new element for the elucidation of the mechanism(s) of action of compounds of this group.

DISCUSSION.—It has been shown in this work that of the fungicides which are believed to act as site-specific inhibitors only those whose site of action is located within the nucleus are capable of inducing somatic segregation in diploid *A. nidulans*. Although compounds 1-5 in Table 1, had not previously been tested for ability to induce sectoring, their ineffectiveness, was expected on the basis of available knowledge on their mechanism of action (7, 18, 22, 26, 29). These compounds were included in the present study in order to demonstrate the reliability of the induced sectoring test and they were found completely devoid of activity, although they are powerful inhibitors of fungal growth (Table 1). The comparison to compounds 6-8 proves that the ability to induce sectoring is a safe criterion to distinguish compounds which inhibit growth by interfering with some hereditary process. This criterion is also a very sensitive one, perhaps more so than growth inhibition itself. Even at concentrations hardly inhibitory to growth, some compounds increased the number of sectors 200-300% (Table 1). It would probably be much more difficult to recognize any effect by cytological examinations in such cases. We suggest that this genetic test could be applied routinely at the early stages of any attempts to elucidate the mechanism of action of a new fungicide.

Of the selective inhibitors of nuclear activity included in Table 1, benomyl (13,17) and griseofulvin (16) had earlier been reported to induce sectoring in diploid colonies. We have seen no similar report for actinomycin D. Other inhibitors of nucleic acid synthesis which probably would induce sectoring in diploid *A. nidulans* are mitomycin C and 5-fluorodeoxyuridine which have been shown to induce mitotic crossing over in other fungi (8,14). It is not known whether actinomycin D increases sectoring also by induction of mitotic crossing over. At low concentrations this antibiotic acts primarily by inhibiting DNA-dependent RNA polymerase but inhibition of DNA synthesis and chromosome breaks may also be caused (see ref. 24). In contrast to mitomycin C, benomyl-and griseofulvin-induced sectoring seems to result primarily from chromosome nondisjunction (16, 17).

Members of the aromatic hydrocarbon group have been reported previously to interfere with nuclear function in higher organisms. Wuu and Grant (31), working with barley root tips, found that dicloran (botran) induces chromosome aberrations; and acenaphthene, also a member of the group (10), has long been known as a polyploidogenic agent (see ref. 15). For some reason, such effects have not received adequate attention as a possible cause of the fungitoxicity of this group of fungicides. Attention has been directed to other effects on fungal cells (9,19,27,30) as mentioned in the previous section. However, while none of these other effects ascribed to an aromatic hydrocarbon fungicide has been shown to also be caused by other members of the group, the ability to induce somatic segregation appears to be a common property of all members (Table 1). Further, the external concentration required for large increases in the number of sectors in the diploid *A. nidulans* are rather low compared to those needed for example to cause chromosome aberrations in barley (31). And since these concentrations are comparable to those at which specific inhibitors of nuclear processes induce similar effects (Table 1), it is reasonable to suggest that the effect of aromatic hydrocarbon fungicides on fungal nuclei is a primary one and that effects on other processes are probably secondary or take place only at high concentrations. It is important to note that an aromatic hydrocarbon nucleus is not sufficient to include a fungicide in the group. Daconil, for example, does not induce sectoring (Table 1), and cross-resistance tests (28) have also indicated that the biological activity of this fungicide is different from that of the aromatic hydrocarbons.

Hammerschlag and Sisler (12) have suggested that the ineffectiveness of griseofulvin and of the benzimidazole-type fungicides against several Oomycetes may be due to the absence of the type of mitotic apparatus which is typical of higher fungi. Some aromatic hydrocarbons, here reported to have a definite effect on nuclear function, also do not inhibit the growth of these lower fungi. Ramsey et al. (23),for example, have reported that diphenyl may even have beneficial effects on the growth of *Phytophthora citrophthora*. Thus action on nuclear processes may also be an important cause of fungicidal selectivity.

Fungal mutants resistant to an aromatic hydrocarbon fungicide are very easy to obtain and such mutants are always cross-resistant to all members of the group (10,28). If the high genetic activity of these compounds (Table 1) is mainly responsible for their fungitoxicity, it is tempting to suggest that mutational modification of some nuclear component would be sufficient to explain resistance to all of the aromatic hydrocarbons. Such an explanation would seem reasonable, particularly since it was found that mutant and wild-type cells do not differ in the amount of fungicide taken up (28). Davidse (6) has evidence that the resistance to benzimidazoles may result from modification of the fungicide-binding capacity of a macromolecule which is probably identical with fungal tubulin.

Although the results obtained in this work are mainly discussed from the point of view of fungitoxic mechanisms, it must be stated that the use of fungicides which affect hereditary processes may have its impacts on other organisms, including man. A very important aspect being studied in several laboratories, is the possible ability of fungicides to induce gene mutations. From the point of view of genetic hazard, however, fungicides possessing genetic activity of any kind should be avoided if nonactive alternatives are available. Fungicides numbered 1-5 and 9-13 in Table 1 are good examples of such nonactive alternatives.

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