Environmental Health Perspectives
Vol. 31, pp. 81-95, 1979

Analysis of Mitotic Nondisjunction with *Aspergillus nidulans*

by G. Morpurgo,* D. Bellincampi,* G. Gualandi,* L. Baldinelli,* and O. Serlupi Crescenzi*

Two methods to detect the induction of nondisjunction with a diploid stable strain of *A. nidulans* are described. The first method gives only qualitative results, while the second method is quantitative and dose-effect curves can be done. Some physiological parameters affecting the induction of nondisjunction can also be studied, because either quiescent or germinating conidia can be treated with the drug under test.

Some agents inducing nondisjunction were also tested for the induction of point mutation and somatic crossing-over with these comparative analysis. Two classes of agents inducing nondisjunction may be detected: the first causes all possible types of genetic damage either on quiescent or germinating conidia (a representative of this class is MMS) and acts presumably on the DNA level; the second acts only on germinating conidia and does not produce point mutation or crossing over. A representative of this class is Benomyl which interferes with spindle microtubules. A list of compounds tests is included.

Introduction

Aspergillus nidulans is an ascomycete fungus widely exploited in genetic research. Its normal condition is haploid but diploid strains can be easily obtained (*1*). The diploid strains are very stable; segregants can rarely occur by two processes; i.e., mitotic crossing over, which leads to recombinant diploid strains, and mitotic nondisjunction. This second process originates an unbalanced aneuploid which can evolve towards a balanced condition either diploid (with one or more chromosome in homozygosis) or haploid (*2*).

Both processes of recombination are rare: the spontaneous frequency of crossing over in a region whose meiotic length is about 40 morgans is about 1×10^{-4} (*3*), while the spontaneous incidence of nondisjunction is about 0.5×10^{-3} per generation.

In the present paper we will describe the methods to test the rate of nondisjunction, either spontaneous or induced, by use of diploid strains of *Aspergillus nidulans,* and the results obtained insofar will be summarized. We shall also discuss the possibility with these methods of knowing how a drug inducing nondisjunction acts at the cellular level.

*Istituto dell'Orto Botanico, Largo Cristina di Svezia, 24, Roma, and Istituto Superiore di Sanit Roma, Italy.

Experimental

The Strain

The genetic map of the first chromosome of the strain P is shown in Figure 1. The strain is also heterozygous for the markers S12; phen A2, meth G1; pyro A4; nicA2; lys B5; nicB8 on different linkage groups. Symbols are from Barrat et al. (*4*).

The strain is green (light green because of the incomplete dominance of the y allele), prototrophic and sensitive to *p*-fluorophenylalanine because all the markers are recessive. With this strain the segregants, nondisjunctional in the first chromosome, must be yellow or dark green, depending which of the two chromosome is in homozygosis or in hemizygosis. The nondisjunctional yellow sectors should also require *p*-aminobenzoic acid and aneurine and will be *p*-fluorophenylalanine-resistant.

Because the first event in nondisjunction is the production of an unbalanced aneuploid, the stable colored nondisjunctional types, either diploid or haploid, will appear as sectors arising from a poorly growing nonsporulated or poorly sporulated colony. As shown by Kafer (*2*), the balanced nondisjunctional types result, from successive events, automatically selected during the growth of the aneuploid which ultimately leads to the formation of the balanced form which is always a stable diploid or haploid.

FIGURE 1. Map of the first chromosome of the diploid strain P of *Aspergillus nidulans*.

Media

Two media are used: Czapek Dox minimal medium contains $NaNO_3$, 3.3 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.01 g; KH_2PO_4, 1 g; $CuSO_4$, 37 g; agar, 20 g; distilled H_2O, 1000 ml; pH 6-6.2; to this is added all the requirements in heterozygous conditions of the strain. The complete medium has the following composition: KH_2PO_4, 1 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.01 g; cornsteep, 10 g; methionine, 0.05 g; yeast extract, 3 g; hydrolyzed nucleic acids, 0.04 g; H_2O, 1000 ml; pH 6.5.

Methods

We routinely use two methods, a plate test and a liquid test, which are discussed separately.

Plate Test. A small number of conidia (about 50) are added to the melted agarized Czapek Dox medium to which the drug to be tested has been added at increasing concentrations. The medium is poured in the dishes and incubated at 37°C. The effective dose (if any) is that which produces the maximum possible inhibition in the growth of the colonies. After 3-4 days, the colonies are transferred with a needle to dishes of complete medium and incubated for three to four additional days. The colonies are then inspected for the presence of yellow or dark green sectors (Fig. 2). Sectors will be further analyzed to test their nutritional requirements. Obviously the evidence of induction of nondisjunction is given by an excess of sectors over the control.

The technique is easy and extremely efficient (provided we have reached the maximum possible inhibitory dose); its defect is that it is impossible to obtain quantitative data. Actually we treat with the drug not a single cell but a whole population of cells of unknown size. It is therefore impossible to establish a really quantitative dose-effect curve.

A probably incomplete list of the compounds tested with this technique (or a very similar technique) in our and in other laboratories can be found in Tables 1 and 2. Ethyl alcohol was first discovered to be a nondisjunctional agent by Harsany et al. (*7*) and their results were confirmed by us. The action of some other compounds is described elsewhere (*5, 6*). The data from other laboratories derive from Kappas, Georgopulos and Hastie (*8, 9*). Very recently we tested one other polyene antibiotic, pymaricin, which efficiently induces nondisjunction. We tested also atrazine, with and without activation by plant cells, with negative effect. We think that some discrepancies in the results between our data and those obtained by others is due to the fact that we use much more inhibitory doses.

Liquid Test. The liquid test can be used either on quiescent or on germinating conidia.

Quiescent conidia, 50,000 conidia/ml suspended in water, are treated with the drug under test at various concentrations for several time with shaking at 37°C (usually some hours but the time of the treatment depends on the toxicity of the drug). Conidia are then plated on the complete medium at the density of 10-15 conidia per dish and incubated at 37°C. After two to three days the dishes are scored to detect the presence of the microcolonies. When present, these are transferred with a needle to other dishes in complete medium. This operation is necessary to avoid the overgrowth of the normal fast growing colonies on the slow growing microcolonies which are possibly aneuploid. After three more days the colonies are examined to detect the presence of large yellow or dark green sectors and their genetic constitution is analyzed.

Germinating conidia are incubated in liquid Czapek Dox minimal medium enriched with all the heterozygous requirements of the strain. The concentration of the conidia must be 50.000/ml or less. The minimal medium is also modified with agar added at the concentration of 0.2/1000 ml. The agarization should be done by melting an agarized medium (concentration 2/1000) and mixing it with the same liquid medium to the final proportion 1 to 10. The agarization is necessary to avoid the clumping of the conidia. After 3 hr of incubation at 37°C in small flasks with gentle shaking, the germination of the conidia is controlled at the microscope observing the initiation of budding. Samples of germinating conidia are then treated with the drug under test at various concentrations for several times at 37°C with shaking, and plated on complete medium at a density of 10-15 per dish. The other operations are identical to those of the previous point.

This method is a little more laborious than the plate method but has the great advantage that nondisjunction is induced on single conidia, thus permitting an exact quantitativeness and the construction of a dose response curve. Still more important is the fact that with parallel treatments on quiescent and germinating conidia it is possible to correlate the

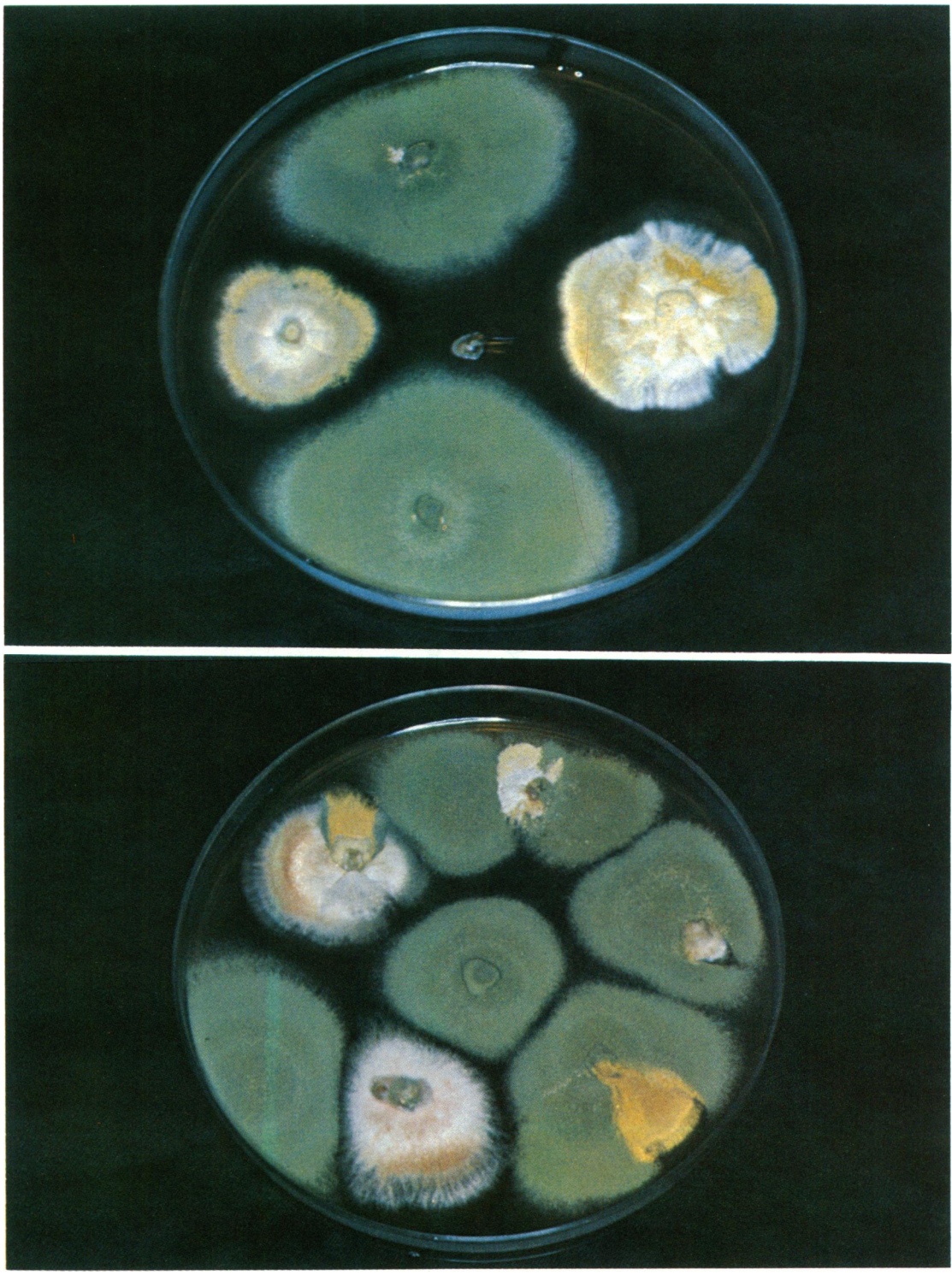


FIGURE 2. Induction of nondisjunctional sectors with *p*-fluorophenylalanine: (*a*) plate showing, at twelve o'clock, a colony showing a nondisjunctional dark green sector requiring riboflavine, proline, and biotine, and a normal sector; at three and nine o'clock, aneuploids colonies, and at six o'clock, a normal diploid colony; (*b*) plate showing, at eleven o'clock, a colony with an aneuploid center from which originates a yellow euploid nondisjunctional sector requiring *p*-aminobenzoic acid, aneurine, and PFP^r and a dark green twin sector requiring proline, riboflavine, biotine, and PFP^s. Other aneuploid sectors from other colonies in the dish (*b*) show segregation of nondisjunctional sectors.

Table 1. Drugs tested for induction of nondisjunction (plate test) in our laboratory.

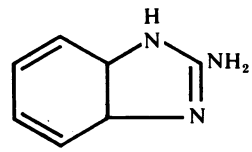
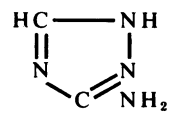
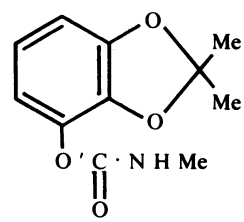
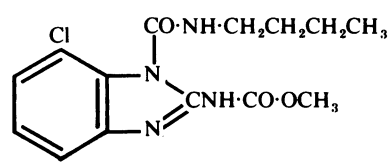
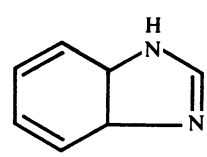
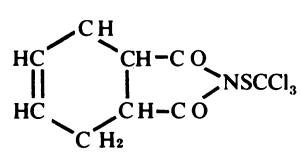
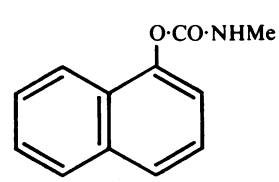
Compound	Chemical structure	Maximum nonlethal dose tested, mg/ml	Effect
Aminobenzimidazole		0.5	–
Aminotriazole		0.4	+
Amphotericin B	Polyene antibiotic	0.005	+
Bendiocarb		0.04	–
Benomyl		0.0002	+
Benzimidazole		0.4	–
Captan		0.04	–
Carbaryl		0.1	–

Table 1 (cont'd).

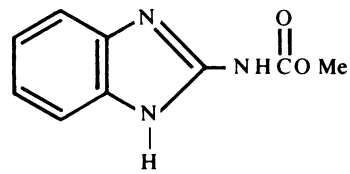
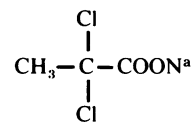
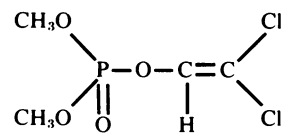
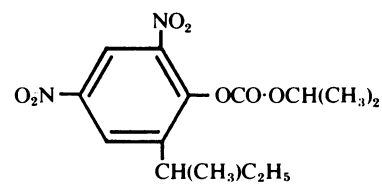
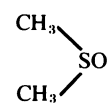
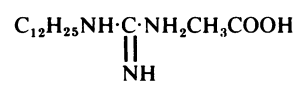
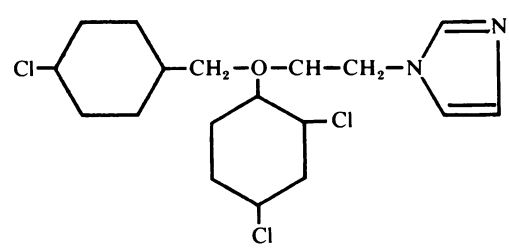
Compound	Chemical structure	Maximum nonlethal dose tested, mg/ml	Effect
Carbendazim		0.00028	+
Dalapon		0.8	–
Dichlorvos		0.8	+
Dinobuton		2.0	–
Dimethyl sulfoxide		4.1	–
Dodine		0.007	–
Econazole		0.004	+
Ethyl alcohol	C_2H_5OH	30.0	+

Table 1 (cont'd).

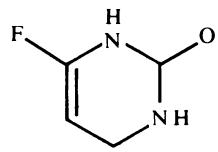
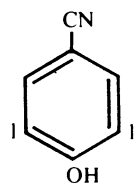
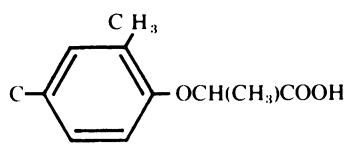
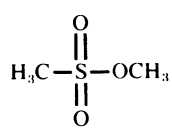
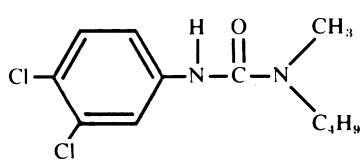
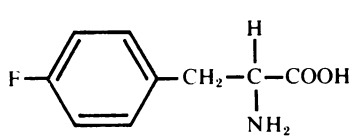
Compound	Chemical structure	Maximum nonlethal dose tested, mg/ml	Effect
Formaldehyde	H C H O	0.02	+
5-Fluorouracil		0.05	–
Ioxynil		0.025	–
Mecoprop		0.35	–
Methyl methanesulfonate			
Methyl urethane	$H_2N-COO-CH_3$	0.4	–
Neburon		1.0	–
p-Fluorophenylalanine		0.036	+

Table 1 (cont'd).

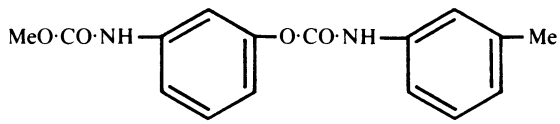
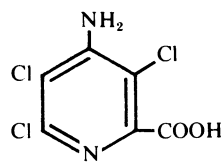
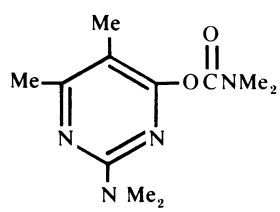
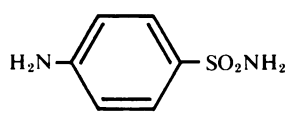
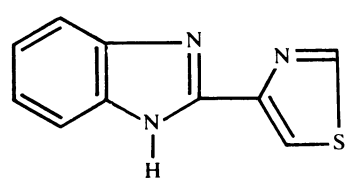
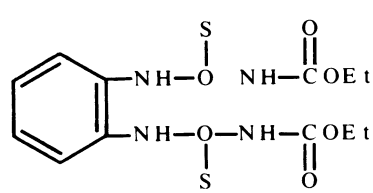
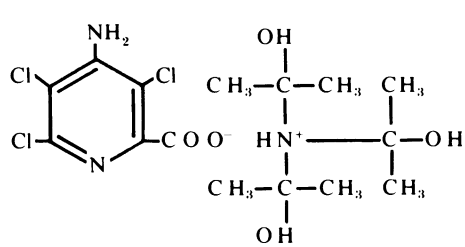
Compound	Chemical structure	Maximum nonlethal dose tested, mg/ml	Effect
Phenmedipham		0.04	+
Picloram		0.8	–
Pirimicarb		0.1	–
Sulfanilamide		0.05	–
Thiabenzazole		0.02	+
Thiophanate		0.1	+
Tordon		1.5	–

Table 2. Drugs tested for induction of nondisjunction (plate test) in other laboratories.

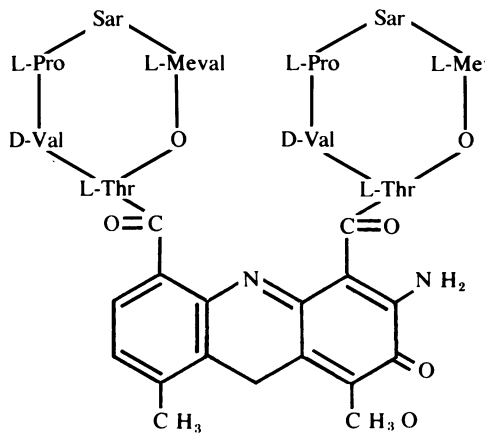
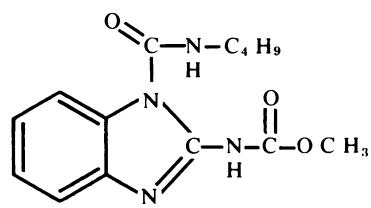
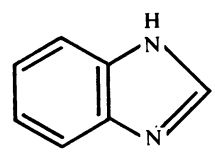
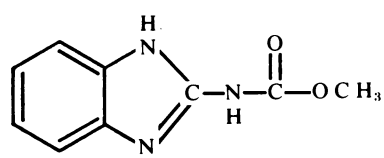
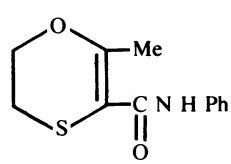
Compound	Chemical structure	Maximum nonlethal dose tested, μM	Effect
Actinomycin D		24	+
Benomyl		1.75	+
Benzimidazole		4000	–
Carbendazim		2.50	+
Carboxin		12	–

Table 2 (cont'd).

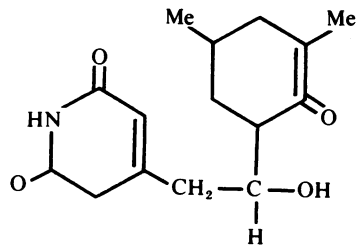
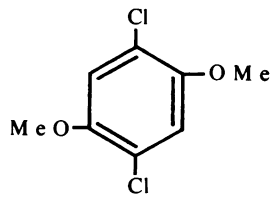
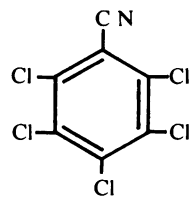
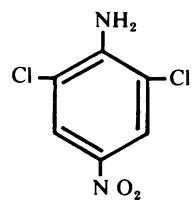
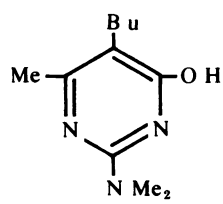
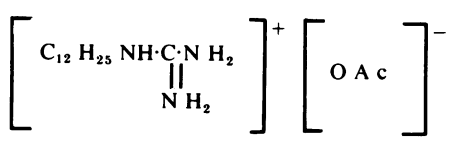
Compound	Chemical structure	Maximum nonlethal dose tested, μM	Effect
Cycloheximide		540	–
Chloroneb		48	+
Daconil		3.0	–
Dicloran		38.0	+
Dimethirimol		4800	–
Dodine		7.0	–

Table 2 (cont'd).

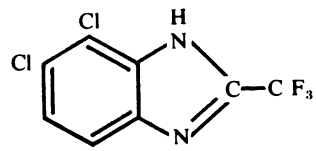
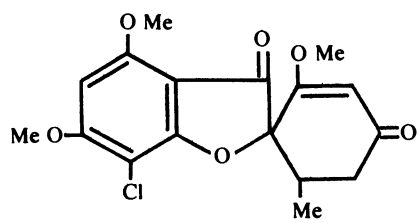
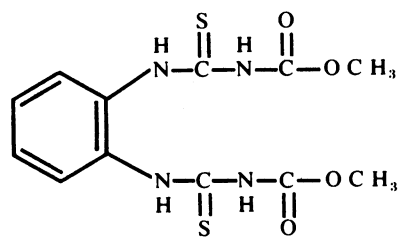
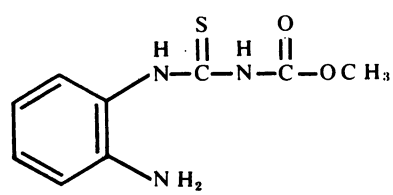
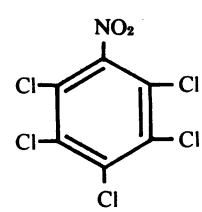
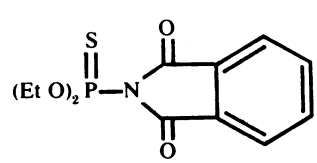
Compound	Chemical structure	Maximum nonlethal dose tested, μM	Effect
DTFB		30.0	–
Griseofulvin		108.0	+
Methyl thiophanate		14	+
2 (-3-Methoxy carbonyl-thioureido aniline)		7	+
Nystatin	Polyene antiobiotic	60	–
Pentachloronitrobenzene (PCNB)		17	+
Plondrel		2500	–

Table 2 (cont'd).

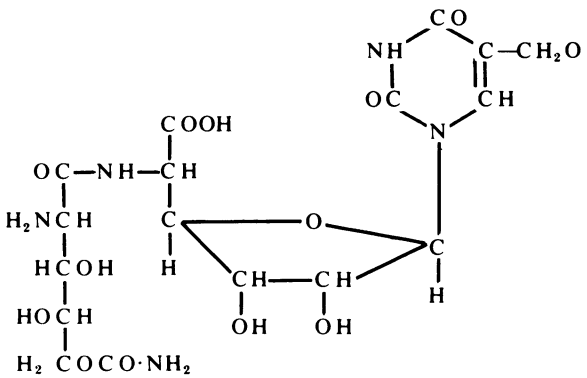
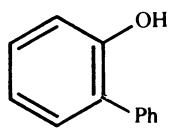
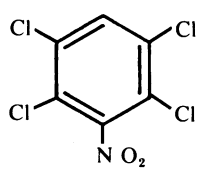
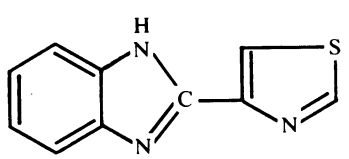
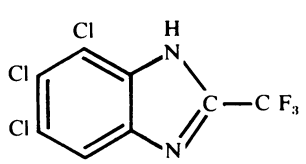
Compound	Chemical structure	Maximum nonlethal dose tested, μM	Effect
Polyoxin D		9	–
SOPP		52	+
Tetrachloronitrobenzene (TCNB)		24	+
Thiabenzazole		10	+
Thiophanate		16	+

Table 2 (cont'd).

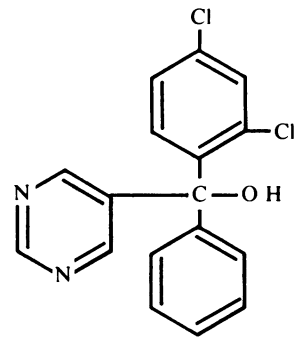
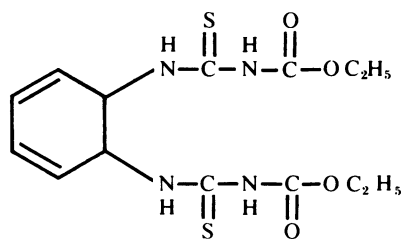
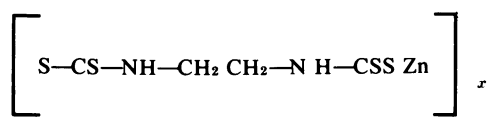
Compound	Chemical structure	Maximum nonlethal dose tested, μM	Effect
Triarimol		30	–
TTFB		28	–
Zineb		1800	–

Table 3

Drug	Nondisjunction		Point mutation	Crossing-over
	Quiescent conidia	Germinating conidia		
MMS	+	+	+	+
4-NQO	+	+	+	+
Nitrogen mustard (HN-2)	+	ND[a]	+	+
Benomyl	–	+	–	–
Ethyl alcohol	–	+	–	ND[a]
p-Fluorophenylalanine	(–)	+	–	–

[a]Not determined.

genetic action with the physiological condition of the cell, thus permitting tentatively to identify the target of the drug in the induction of nondisjunction. The rationale of the system is the following. The two main targets in the induction of nondisjunction are evidently the DNA and the mitotic spindle. Drugs acting on DNA should be in most cases: (1) active on quiescent as well as on germinating conidia; (2) active in inducing point mutations and eventually crossing-over and gene conversion. On the contrary, drugs acting on the spindle or in general outside of DNA should be: (1) inactive on quiescent conidia; (2) inactive in inducing point mutation, crossing over, and gene conversion.

Table 3 summarizes the results we have obtained following this line of research. The data clearly show that the classical mutagens directly alkylating DNA are positive in all tests while the drugs presumably not acting on DNA are active only in inducing nondisjunction in germinating conidia.

Actually it is known that Benomyl (*11*) and ethanol (*7*) interfere with the spindle fibers. It is also proba-

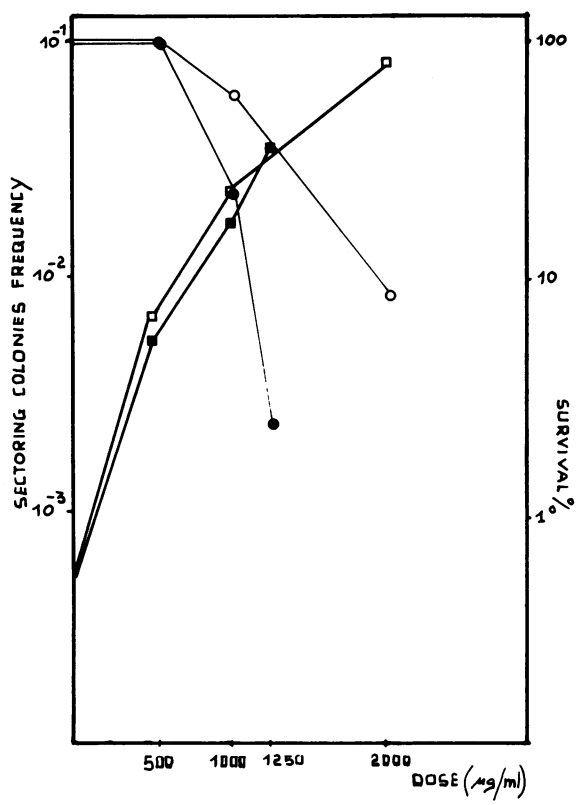


FIGURE 3. Dose-response curves obtained with (○, □) germinating conidia and (●, ■) quiescent conidia on MMS: (○, ●) survival; (□, ■) frequency of nondisjunction. The data are from Gualandi et al. (*11*).

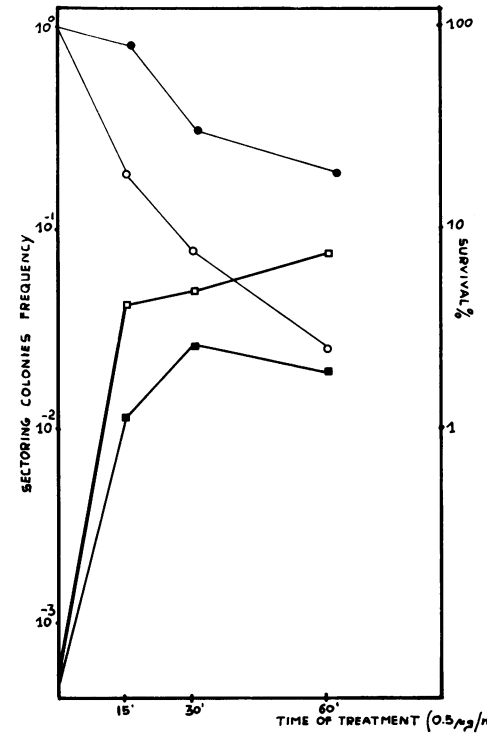


FIGURE 4. Dose-response curves with 4-nitroquinoline *N*-oxide. Symbols as in Fig. 1.

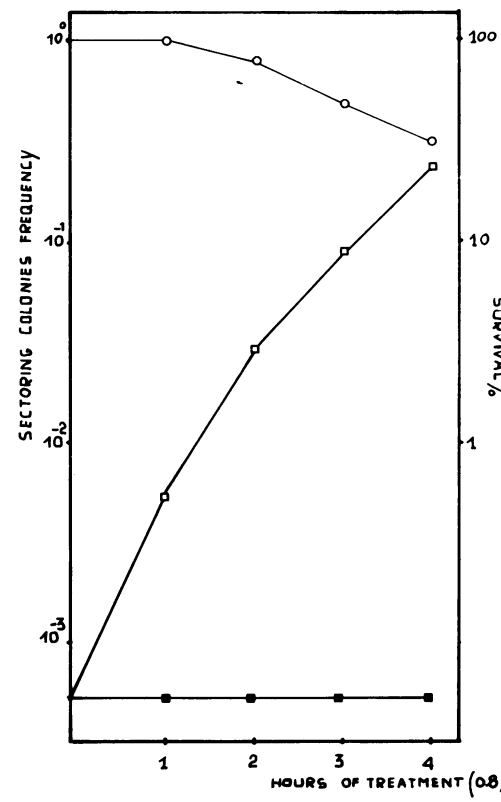


FIGURE 5. Dose-response curves with Benonyl. Symbols as in Fig. 1.

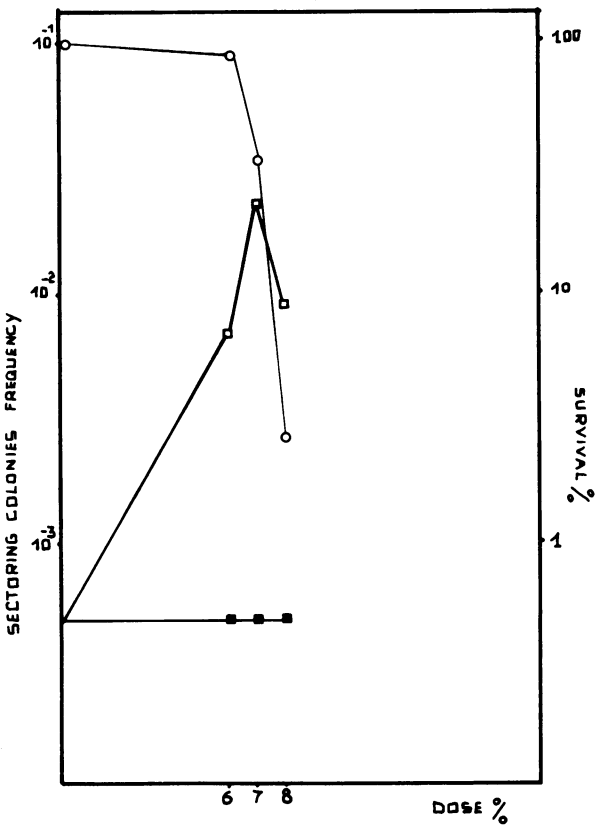


FIGURE 6. Dose-response curves with ethyl alcohol. Symbols as in Fig. 1.

ble that *p*-fluorophenylalanine acts in the same way. *p*-Fluorophenylalanine is an analog of phenylalanine which is incorporated into the proteins and may cause serious disturbances to their functioning.

In Figures 3-6 dose response curves on quiescent and germinating conidia for methyl methanesulfonate (MMS), 4-nitroquinoline *N*-oxide, Benomyl, and alcohol are reported.

Discussion

The data here reported show that induction of nondisjunction can be easily studied by using diploid strains of *Aspergillus nidulans*. The system that we have developed permits an efficient and quantitative analysis of the process.

The available data also suggest that a comparative analysis of all the induced genetic damage, i.e. nondisjunction, point mutation, and crossing over in quiescent and germinating conidia, can demonstrate whether the nondisjunctional agent works on the DNA or at the cytoplasmic level, possibly on the mitotic spindle.

The most important problems now necessary to be solved and of great relevance in the environmental mutagenesis are: the extrapolation to the superior organisms of the data obtained with Aspergillus and the investigation of the existence of a possible threshold in the induction of nondisjunction, either on the agents working on the spindle or on those working on DNA.

It is particularly difficult to obtain data on the first problem: in order to get a partial answer to the problem we are now planning to correlate the action of the agents inducing nondisjunction in *Aspergillus nidulans*, presumably working on the spindle with the block in metaphase on cultured cells of mammals.

We have also preliminary evidence that antibiotics like amphotericin B and pimarcin, acting on the membranes, can induce nondisjunction in Aspergillus; we are now planning to extend these data to other agents damaging the membrane function and to study if similar effects can be observed on the cells of higher organisms. These data are at variance with those of Georgopulos et al. (*9*). The discrepancy is due to the fact that we use doses much more inhibitory than do these authors.

This work was carried out in part under contract 177-77-1 ENVI of the E.C. Environmental Research Program to Prof. Angelo Carere of the Istituto Superiore di Sanità and in part by Consiglo Nazionale delle Richerche (C.N.R.) p.f. Promozione delle Qualità dell'Ambiente.

REFERENCES

1. Pontecorvo et al. The genetics of *Aspergillus nidulans*. Adv. Genet. 5: 141 (1953).
2. Kafer, E. The processes of spontaneous recombination in vegetative nuclei of *Aspergillus nidulans*. Genetics 46: 1581 (1961).
3. Morpurgo, G. Induction of mitotic crossing-over in *Aspergillus nidulans* by bifunctional alkylating agents. Genetics 48: 1259 (1963).
4. Barrat, R. W., Ogata, W. N., and Kafer, E. Aspergillus stock list (1st Revision July 1975). Aspergillus News Letter 13: 23 (1975).
5. Bignami, M., Aulicino, F., Veleich, A., Carere, A., and Morpurgo, G. Mutagenic and Recombinogenic action of pesticides in *Aspergillus nidulans*. Mutat. Res. 46: 395 (1977).
6. Fratello, B., Morpurgo, G., and Sermonti, G. Induced somatic segregation in *Aspergillus nidulans*. Genetics 45: 785 (1960).
7. Harsany, Z., Granek, I. A., and Mackenzie, D. W. R. Genetic damage induced by ethyl alcohol in *Aspergillus nidulans*. Mutat. Res. 48: 51 (1977).
8. Kappas, A., Georgopulos, S. G., and Hastie, A. C. On the genetic activity of benzimidazole and thiophanate fungicides on diploid *Aspergillus nidulans*. Mutat. Res. 26: 17 (1974).
9. Georgopulos, S. G., Kappas, A., and Hastie, A. C. Induced sectoring in diploid *Aspergillus nidulans* as a criterion of fungitoxicity by interference with hereditary processes. Phytopathol. 66: 217 (1975).

10. Davidse, L. C. The antimitotic activity of methyl-benzimidazol-2-yl carbamate in fungi and its binding to cellular protein. In: Microtubules and Microtubule Inhibitors, M. Bargers and, M. De Brabander. Eds., North Holland, Amsterdam, 1975, pp. 483-495.
11. Gualandi, G., Bellincampi, D., and Puppo, S. MMS induction of different types of genetic damage in *Aspergillus nidulans:* a comparative analysis in mutagenesis. Mutat. Res., in press.
12. Morpurgo, G. Quantitative measurements of induced somatic segregation in *Aspergillus nidulans*. Sci. Repts. Ist. Sup. Sanità 2: 234 (1962).