DEVELOPMENT AND STANDARDIZATION OF
A MUTAGENICITY TEST SYSTEM USING
ASPERGILLUS NIDULANS AS TEST ORGANISM

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DEVELOPMENT AND STANDARDIZATION OF A MUTAGENICITY TEST SYSTEM USING ASPERGILLUS NIDULANS AS TEST ORGANISM

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
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des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

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STELLINGEN

I

Het is het overwegen waard om in aanvulling op andere tests Aspergillus nidulans te gebruiken als testorganisme voor het aantonen van mutageniteit.

II

Bij langdurige inkubatie in aanwezigheid van de te onderzoeken stof kunnen, ook bij andere testorganismen dan Aspergillus nidulans, selektie effekten optreden die aangezien kunnen worden voor mutagene eigenschappen.

III

Voor de evaluatie van eventuele mutagene eigenschappen van fungiciden zijn de resultaten van tests met schimmels als testorganismen minder zwaarwegend dan de resultaten van tests met anderssoortige testorganismen.

IV

In hun onderzoek naar deleties van een duplikatie stam houden Nga en Roper te weinig rekening met het optreden van crossing-overs.

B.H. Nga en J.A. Roper, 1968. Genetics 58:193-209

V

De isolatie van carbendazim resistente kolonies kan niet gebruikt worden om de mutageniteit van dezelfde stof te bewijzen.

H.I. Nirenberg en J.B. Speakman, 1981. Mut. Res. 88:53-59

VI

Een te grondige verwijdering van mineralen uit het water dat als basis dient voor het vervaardigen van voedingsmedia kan komplikaties veroorzaken.

VII

Bij gebruik van wilde en primitieve soorten in de veredeling van voedselgewassen zou men ook onderzoek moeten doen naar eventuele overgedragen mutagene eigenschappen.

VIII

Als in het laboratorium het glaswerk op dezelfde manier gesteriliseerd zou worden als in het huishouden de flessen voor babyvoeding dan zouden veel proeven mislukken.

IX

Fietspaden -zoals men ze in Duitsland vaak aantreft- waarop bij elke kruising een stoep van ca. 5 cm voorkomt, vertragen de doorstroming van het verkeer.

X

Met de term "buitenlanders" wordt in de omgangstaal vaak niet zozeer een verschil in nationaliteit aangeduid maar wel een kultureel verschil.

XI

Het uitgeven van mooie strafportzegels lokt het versturen van ondergefrankeerde brieven uit.

Proefschrift van J.G. Boschloo, getiteld: Development and standardization of a mutagenicity test system using Aspergillus nidulans as test organism.
Wageningen, 29 mei 1985.

VOORWOORD

Aan de totstandkoming van dit proefschrift hebben meerdere personen hun bijdrage geleverd.

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Andrea Mittelstädt hat im Rahmen ihrer Diplomarbeit einige der in dieser Dissertation beschriebene Versuche durchgeführt.

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CONTENTS

LIST OF ABBREVIATIONS AND MOST IMPORTANT GENE SYMBOLS:

9AA	9-aminoacridine
ace	acetate
acr	acriflavine resistance marker
ACR	acriflavine
ad	adenine marker
ade	adenine
an	aneurine / thiamine marker
ara	arabinose
BED	butadienediepoxide
bi	biotin marker
bio	biotin
CH	chloralhydrate
cha	chartreuse marker
cho	choline marker
cho	choline
CM	complete medium
c.o.	crossing-over
cre	carbon derepression marker
des	desoxycholate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dp	duplication
fac	fluoroacetate resistance and acetate non-utilizing marker
fpa	para-fluorophenylalanine resistance marker
FPA	para-fluorophenylalanine
fw	fawn marker
gal	galactose non-utilizing marker
glu	glucose
lac	lactose non-utilizing marker
lys	lysine
m	multiplying effect on the spontaneous mutant frequency caused by mutagenicity
MBC	carbendazim
MEA	malt extract agar
meth	methionine marker
meth	methionine
MM	minimal medium
MMS	methylmethanesulfonate
N3	sodium azide
n.d.	non-disjunction
nic	nicotinic acid
NO2	sodium nitrite
paba	para-aminobenzoic acid marker
paba	para-aminobenzoic acid
pdh	pyruvate dehydrogenase deficiency marker
phen	phenylalanine marker
phen	phenylalanine
pim	pimaricin resistance marker
PIM	pimaricin
pro	proline marker
pro	proline
pyro	pyridoxin marker
pyro	pyridoxin
ribo	riboflavin marker
ribo	riboflavin
s	multiplying effect on the spontaneous mutant frequency caused by selective advantage
s.d.	standard deviation
SM	supplemented medium
sor	sorbose resistance marker
SOR	sorbose
suad	suppressor adenine marker
thi	thiamine
VIN	vinclozolin
y	yellow marker
w	white marker

1 INTRODUCTION

1.1 General introduction

The continuous introduction of new chemicals into the environment is a problem of growing concern. One of the possible hazards is their potential to induce mutations. But even naturally occurring substances which have been used for years and are regarded as save, may have strong mutagenic properties (Ames, 1983).

Mutations are heritable changes in the composition or arrangement of genes, or in the structure and number of chromosomes. They may be transmitted from one cell generation to another within an individual (somatic mutations) and in some cases to the progeny of the affected individual (germinal mutations). Mutations can result in genetic diseases. The total effect of a mutagenic exposure of a population will only be revealed after a long period of time, since many mutations will first be expressed in the individuals of the following generations. Thus there will be a pronounced delay in monitoring mutagenic activity (Carter, 1977).

Somatic mutations may be considered as one cause of cancer. Although the exact mechanism of carcinogenesis is not yet known, it has been found that most carcinogens are mutagens as well (McCann et al., 1975). Recent investigations have shown that, at least in one system, a point mutation can change a "normal" cell into a cancer cell (Reddy et al., 1982).

The introduction of new chemical compounds which are mutagenic may augment the frequency of genetically based ill health. In order to estimate the potential hazards of new chemicals, it is necessary to identify whether they are mutagenic or not. Since direct measurement of mutagenicity or carcinogenicity on humans is not possible, many rapid screening tests have been developed (Hollstein et al., 1979), but only few have been thoroughly evaluated. The results of such a test are only valid for the organism and the genetic end-point investigated and give only an indication for other situations. Thus different tests have to be performed in order to predict the effect of a specific chemical compound on men. Generally, the use of mammals as test

organisms or human cell cultures will have better predictive value than the use of micro-organisms, but the costs of such tests will be higher. Therefore a battery of test systems has been recommended, including different genetic end-points and different organisms varying from bacteria to mammals (Anonymous, 1980).

1.2 Aspergillus nidulans as a test organism for mutagenicity testing

One possible organism for mutagenicity testing is Aspergillus nidulans. Being a lower eukaryot, it combines some advantages of bacterial test systems with those of higher organisms. This organism has a colonial growth on agar solidified medium and forms an abundance of vegetative spores (conidia). Its genetic system has been examined in detail; since it is possible to grow this organism in haploid (normal form, n = 8 chromosomes) as well as in diploid condition, not only point mutations can be studied but also other genetic end-points like enhanced mitotic crossing-overs, non-disjunctions or chromosomal rearrangements.

In view of the potential value of Aspergillus for the screening of genetic effects, several tests with this organism have been developed.
-A- Haploid strains are used for the detection of point mutations (For a more detailed review, see Scott et al., 1982):
-A1- Most generally used is the system in which revertants of the mutant methG1 allele are counted. Revertants which grow on media lacking methionine can be divided into 3 classes on the basis of their different morphology (Lilly, 1965). All 3 classes contain or are thought to contain mutants at at least 2 loci (Lilly, 1965; Scott & Alderson, 1971). The genetic analysis of 9 mutants showed that they all were suppressortype mutants.
-A2- Another possibility for the detection of point mutations is the 2-thioxanthine system. Wildtype Aspergillus strains produce green conidia on normal media. But when the medium used contains 2-thioxanthine, the conidia have a yellow colour. After transport into the cells, 2-thioxanthine is converted into 2-thiouric acid by the enzyme xanthine dehydrogenase and this conversion leads to a yellow pigment. Mutants lacking xanthine dehydrogenase or the transport system for 2-thioxanthine will have normal green conidia, whether 2-thioxanthine

is present in the medium or not. After isolation, mutants can be assigned to several classes if they are tested for growth on different nitrogen sources. Complementation analysis has shown that about 10 loci are involved (Alderson & Hartley, 1969).
-A3- A Russian group uses an arginine requiring strain, and revertants are scored on media lacking arginine (Panchenko, 1974).
-A4- Forward mutations for resistance to toxic chemicals can also readily be used. Bignami et al. (1977) use a system in which resistance to 8-azaguanine is scored.

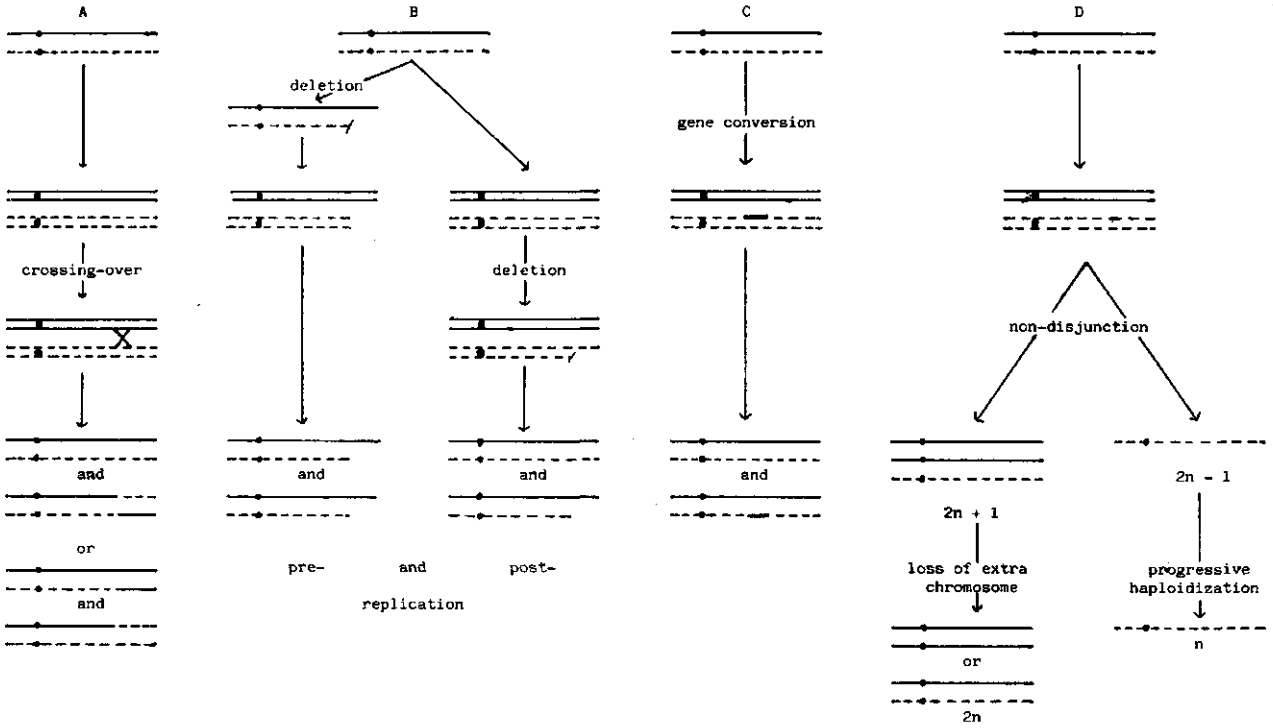
-B- With diploid strains, heterozygous for several recessive genes, colour markers among them, other genetic end-points can be investigated. During cell division (mitosis) in such strains, several processes can lead to the occurrence of sectors with non-parental genotypes.

Most sectors arise by a mitotic crossing-over between the (colour) marker and the centromere. In one of two cases all genes distal to the point of exchange will become homozygous and recessive genes will be expressed (fig 1A). Deletions, however, can also lead to phenotypically similar recombinants (fig 1B). Although in such a diploid the genome is unbalanced, the morphology and growth rate is not necessarily affected (Kappas, 1978). A definite proof of a mitotic crossing-over is only obtained if both reciprocal crossing-over products can be scored. Twin-spots are found. These are neighbouring sectors in which the recessive genes of the 2 homologues are expressed (Wood & Käfer, 1967).

Apart from the reciprocal exchange of genetic material during mitosis, there is also gene conversion: unequal recovery of genetic markers in the region of exchange during genetic recombination. In this process a (very) small piece of DNA of one strand is made complementary to the strand of the homologue (fig 1C). The resulting heteroduplex can be repaired leading to the expression of a recessive gene.

Non-disjunction is a process in which the chromosomes are not equally distributed during anaphase (fig 1D). At first aneuploids are formed ($2n + 1$ and $2n - 1$) which are unstable in Aspergillus. After subsequent loss of one or more chromosomes, they produce stable diploid or haploid colonies. In diploid non-disjunction products in one of three cases the recessive genes of the chromosome involved in the initial non-disjunction will be expressed. Haploids, in which recessive

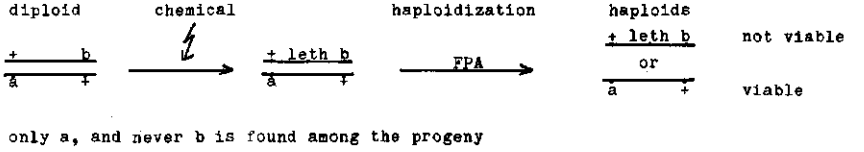
Fig 1 Schematically 4 processes are shown which can lead to homozygosity in a heterozygous diploid during mitosis. A: crossing-over, B: deletion, C: gene conversion, D: non-disjunction. Only the 2 homologues of one chromosome are shown, one as a dotted line and the other as a solid.



genes on all chromosomes are expressed, arise after a non-disjunction of any of the 8 chromosome-pairs. Therefore haploids will be found more frequently than diploid non-disjunction products when a given marker is scored.

An entirely different genetic end-point can also be measured in diploid strains: recessive lethal damage. If a chemical induces a lethal effect which does not come to expression in the target cell itself but only in its haploid progeny, the chemical must have disturbed a vital gene-function on one of the homologues of a chromosome. The damage involved may be a point mutation, a deletion or a structural change in the chromosomes.

In diploid Aspergillus strains with suitable markers, recessive lethal damage can be shown when these diploids are forced to haploidize by means of chemicals like p-fluorophenylalanine (FPA). The absence of a certain marker in the haploid sectors indicates that a recessive lethal must be located on the same chromosome as this marker. Schematically:



only a, and never b is found among the progeny

Several procedures and strains for measuring the frequency of mitotic recombination processes have been used. See Käfer et al. (1982) for a general review. In principle, the procedures used are as follows:
-B1- Inoculation of mycelium of the test strain on mutagen-containing plates of complete medium. About 5 colonies per plate are tested and sectors expressing recessive colour markers are scored after incubation for several days at 37 °C. Isolation of the sectors and subsequent testing for other markers can reveal which mechanism has been responsible for the occurrence of these sectors, if enough markers are present on the chromosome(s) involved (Kappas et al., 1974).
-B2- Another procedure is to incubate the conidia with the mutagen in a liquid suspension and, after separating the conidia from the mutagen, the conidia are plated on complete medium. Normal colonies with recessive coloured sectors and colonies with an abnormal morphology are scored. The abnormal colonies are isolated and replated in order to investigate whether they are aneuploids or stable abnormal colonies.

The sectors can be isolated and tested for markers to reveal the inducing mechanism (Bellincampi et al., 1980).
-B3- Morpurgo et al. (1979) used a system in which the tester strain carries the heterozygous recessive allele for resistance to p-fluorophenylalanine (FPA). After incubation of conidia with mutagen in liquid suspension, these conidia are plated on complete medium containing FPA and the mutagen (Bignami et al., 1974). After incubation, recombinant colonies are scored. Since they use a strain with a yellow colour marker located on the same chromosome as the fpa allele but at the other side of the centromere, they can distinguish directly mitotic crossing-over products (green colonies) from non-disjunction products (yellow colonies).

An analogous system has been developed using the gene for pimaricin resistance (Bertoldi et al., 1980).

A complication is that both FPA and pimaricin are reported to induce non-disjunctions themselves (Lhoas, 1961; Bellincampi et al., 1980), and when a positive effect on the number of non-disjunctions is found, this result must be verified by another, non-selective test method (Bignami et al., 1974).

The gene conversion frequency is very low in Aspergillus and therefore cannot be measured with the procedures mentioned before.
-B4- One system for estimating the gene conversion frequency with a diploid strain, heterozygous for 2 pabaA (para-aminobenzoic acid requiring) alleles in trans-position, has been described (Bertoldi et al., 1980). The spontaneous frequency of paba prototrophs when the pabaA1 and pabaA6 alleles are involved is 1.4×10^{-6} prototrophs/colonies. Investigations of Bandiera et al. (1973) showed that at least a part of the paba prototrophs arise after a gene conversion process.

Several strains have been used to score recessive lethal damage.
-B5- Azevedo (1970) used a strain with markers on all chromosomes, and after chemical treatment many colonies were screened by looking for the presence of all markers among the haploid descendents.
-B6- Azevedo and Roper (1967) looked only for certain, easy to score, markers (conidial colour or morphological mutants) among the haploids.
-B7- Morpurgo et al. (1978) used a strain, heterozygous for a FPA resistance allele, and after haploidization by FPA, the presence of

good sporulating sectors has been scored. For only haploids having the chromosome carrying the FPA resistance allele can grow out to give good conidiating sectors.

-C- Duplication strains can also be used to measure the genetic activity of a chemical, for they are slightly unstable at mitosis, and certain chemicals have been shown to increase this instability (Birkett & Roper, 1977). In a duplication strain, part of a chromosome is present twice in the otherwise haploid strain. One part is in normal position, the other part is present as a translocation connected to the end of another chromosome. Duplication strains are "crinkled": the colonies are smaller than those of the wildtype strains and have a typical morphology (Bainbridge & Roper, 1966). Spontaneously sectors occur, some of which have a normal wildtype morphology. The exact mechanism is not known. Nga and Roper (1969) proposed the term: mitotic non-conformity. Deletion and/or crossing-over processes may play a role.

Different duplication strains are known, but usually the I→II duplication, originally isolated by Pritchard (1960), is used to test chemicals, since in this system the marker for yellow conidial colour can be used for selection.

The procedures used show some variation.
-C1- After mutagen treatment, 1 colony is grown on a plate with complete medium, or 1 colony is inoculated on mutagen-containing complete medium. After an incubation of a week at 37 °C, the number of yellow sectors is counted (Roper et al., 1972; Majerfeld & Roper, 1978).
-C2- After mutagen treatment, 300 - 400 conidia are inoculated on a plate complete medium (supplemented with desoxycholate to restrict colony size) and after an incubation of several days at 37 °C, the number of yellow sectors is counted (Normansell & Holt, 1979).

1.3 Aim and outline of this study

The aim of this study was to explore Aspergillus nidulans in order to come to a comprehensive test system for mutagenicity testing. This test system should include different genetic end-points like mitotic

crossing-over, non-disjunction and point mutation. We preferred selective systems in which all surviving conidia have to be grown and screened after mutagenic treatment. To avoid complications, it has been preferred to use only selective media without chemicals which are known to be mutagenic themselves.

It has been intended to standardize and evaluate the optimal test system by testing several chemical compounds.

2 MATERIALS AND METHODS

2.1 Strains

Several strains of Aspergillus nidulans (Eidam, Winter), which all originally descend from a single Glasgow wildtype isolate, were used in this study (table 1). Aspergillus nidulans is the name for the vegetative form of the Ascomycete Emericella nidulans. The name Aspergillus will be used because the fungus is well-known by this name. The genetic notation used is by Clutterbuck (1981) and Käfer et al. (1982).

Table 1 Strains of Aspergillus nidulans

	number	source	genotype
Haploids:	R002	Roper	proA1 pabaA6 yA2; Dp(I→II) adE20 biA1.
	003	213 x 145	sorA2; wA3; pyroA4.
	005	213 x 475	fpaB37 galD5 suAladE20 riboA1 anA1 pabaA1 yA2 adE20 biA1 sorA2; sD85 fwA2.
	007	002 x 003	proA1 pabaA6 yA2 sorA2; Dp(I→II) adE20 biA1.
	WG008	Wageningen	biA1; acrA1.
	WG096	Wageningen	yA2 pabaA6.
	G110	Glasgow	biA1; methG1.
	WG141	Wageningen	pdhA268(ts); wA3; pyroA4.
	WG145	Wageningen	wA3; pyroA4.
	HA213	Arst	sorA2; adH23.
	HA216	Arst	$creA^{d}1$ pabaA1.
	FG470	FGSC	proA1 biA1; methG1 frA1 T1(IV;VII); chaA1.
	FG473	FGSC	sulA1 adE20; acrA1 wA3; actA1; pyroA4; facA303; lacA1 sB3; oliA2 choA1; riboB2 chaA1.
	FG475	FGSC	fpaB37 galD5 suAladE20 riboA1 anA1 pabaA1 yA2 adE20 biA1; sD85 fwA2.
Diploids:	D1	473/475	see haploid strains
	D3	Morpurgo	anA1 + pabaA1 yA2 + + suAladE20 riboA1 + proA1 + + adE20 biA1; acrA1 + ; phenA2 + ; methG1 + pyroA4 + ; fpaA2 T1(I;V) + nicA2 + + lysB5 + ; nicB8 + .
	D4	005/473	see haploid strains
	D7	de Bertoldi	pimB10 + proA1 + pabaA1 + yA2 + + biA1 pyroA4.

FGSC = Fungal Genetic Stock Centre (California)

2.2 Culture media

2.2.1 Minimal medium

As a basic medium for selection plates and for test plates, a minimal medium (MM) was used. MM contained per 1000 ml distilled water: 6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 15 g agar (Difco) and some cristals of $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuCl_2$. The pH was adjusted to 6.0 by adding NaOH.

The medium was put into flasks and autoclaved (20 min, 120°C and 1 at). Before plates were poured a sterile carbon source and, if necessary, growth factors and amino acids were added. These were all autoclaved separately.

Usually 0.05 M glucose (glu) was used as a carbon source. But in testing colonies for sorbose resistance (sorbose is a pentose which inhibits the growth of Aspergillus on weak carbon sources), 0.1 M acetate (ace), pH 6.0, had to be used. Surprisingly, after sodium azide treatment and subsequent washing of the conidia (incubation method 2, see 3.2), growth on acetate as a carbon source was inhibited. In this case 0.05 M arabinose (ara) replaced the acetate, and colonies could be screened for sorbose resistance.

In the strains D1 and D4, recessive markers for galactose (gal) and lactose non-utilizing (lac) and fluoroacetate resistance / acetate non-utilizing (fac) are present. When segregants of these strains had to be tested for these markers, MM plates were used in which 0.05 M galactose, 0.025 M lactose or 0.1 M acetate, respectively, replaced the glucose.

Usually MM was also supplemented with growth factors and / or amino acids. All test strains carry several markers which make the strain auxotroph for growth factors and / or amino acids. These markers made it possible to distinguish between the different types of segregants.

When mutants or recombinants were expected to be homozygous for such a marker, the growth factors and / or amino acids involved had to be added to the MM. Later on, after isolation, the mutants or recombinants could be tested for the presence of those markers by comparing the growth on MM plates in which one growth factor or amino acid was omitted to the growth on MM plates in which all growth factors and amino acids were present.

The concentrations of the growth factors and the abbreviations used were: 63 µM adenine (ade), 0.016 µM biotin (bio), 14 µM choline (cho), 10 µM pyridoxin (pyro), 2.7 µM riboflavin (ribo), 0.059 µM thiamine (thi) and 8.2 µM nicotinic acid (nic). Amino acids were added in higher concentrations: 1 mM methionine (meth), 0.87 mM proline (pro) and 2.5 mM lysine (lys). All these growth factors and amino acids were autoclaved separately and stored at 4°C.

Another category of markers are the resistance genes. The growth of normal wildtype Aspergillus colonies is inhibited in the presence of the chemical concerned, but when a resistance gene is being expressed, colonies can grow. The following concentrations were used in testing these resistance markers: 0.23 mM acriflavine (ACR), 1.1 mM para-fluorophenylalanine (FPA) and 5.6 mM sorbose (SOR) in acetate medium. The resistance to sorbose was also used in the selection of recombinants of the strains D4 and 007. In this case a higher concentration of sorbose was used (18 mM) since a smaller colony size (of the wildtype colonies but to a lesser extent also of the recombinants) is advantageous here.

2.2.2 Complete medium

For good growth and sporulation of all strains, a complete medium (CM) was used. CM consisted of all ingredients of MM (see 2.2.1) plus, per 1000 ml: 2 g neopeptone, 1 g casamino acids, 1 g yeast extract (all 3 from Difco), 0.3 g RNA (from Sigma) and 2 ml vitamin solution. This vitamin solution contained per 1000 ml: 100 mg riboflavin, 100 mg nicotinic acid, 50 mg pyridoxin, 10 mg thiamine, 10 mg panthothenic acid and 0.2 mg biotin.

CM was, like MM, put into flasks and autoclaved. Prior to pouring plates, 0.05 M glucose was added. Growth factors and amino acids were not needed, but 1 mM desoxycholate (des) was added when a reduced colony size was profitable, as was the case in plates used for viability counts.

2.2.3 Malt extract agar

As a sporulation medium, malt extract agar (MEA) was used. This medium contained per 1000 ml: 20 g malt extract, 1 g neopeptone and

15 g agar (all 3 from Difco). The pH was adjusted to 6.0 and, like the former media, MEA was put into flasks and autoclaved. Glucose (0.05 M) was added as a carbon source. Compared to CM, MEA generally gives a better sporulation of the strains, but, like MM, growth factors and amino acids have to be added separately, so it is less suitable for general use.

MEA was used to grow conidia of the test strains whenever conidial suspensions for mutagenicity testing was required. The occurrence of recombinants (of the diploid strains) could be reduced to a minimal level, since only those growth factors and amino acids needed for growth of the wildtype were added.

MEA supplemented with glucose, growth factors, amino acids and 5.6 µM pimaricin (PIM) was used in selecting pimaricin resistant recombinants of strain D7.

2.2.4 Modified Czapex Dox medium

Since the use of CM in scoring recessive lethals (see chapter 7) was not satisfactory, modified Czapex Dox medium was used. The medium contained per 1000 ml distilled water: 2.55 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4$, 0.037 mg $CuSO_4$ and 15 g agar (Difco). The pH was adjusted to 6.0. Like all former media, this medium was put into flasks and autoclaved. Prior to pouring plates glu, FPA and the growth factors and amino acids needed were added.

2.3 Chemicals tested for mutagenic activity

Several chemicals were tested with the test systems, most of them are well-known mutagens.

The most active mutagens tested are the alkylating agents methylmethanesulfonate (MMS) and DL-1,3-butadienediepoxide (BED), both from Aldrich. MMS is monofunctional and BED has two functional groups. Alkylating agents mainly induce point mutations, but also other kinds of genetic damage (Ehrenberg & Hussain, 1981; Freese, 1971) and are carcinogenic. Special care has been taken in handling these chemicals. All glassware possibly contaminated with MMS was left overnight in a 10% sodium thiosulphate solution, and BED contaminated glassware was left overnight in concentrated HCl. Thus all residues of these

agents had disappeared before the normal cleaning procedures took place.

Sodium azide (NaN_3, N3 for short), from Merck, is a strong mutagen in several systems, especially for lower organisms. The exact mechanism, however, is not known. Often a low pH is needed. N3 is not carcinogenic (Kleinhofs et al., 1978).

Sodium nitrite ($NaNO_2$, NO2 for short), from Merck, is also strongly mutagenic to several systems when tested at a low pH. NO2 acts by deamination (Zimmermann, 1977). NO2 itself is not carcinogenic.

Acriflavine (ACR), from EGA, a commercial mixture of proflavine and trypaflavine, and 9-aminoacridine (9AA), from Fluka, are acridine derivates and induce (frameshift) mutations in several organisms and chromosome aberrations. The carcinogenicity data are inconclusive (Nasim & Brychy, 1979).

Both para-fluorophenylalanine (FPA), from Sigma, and chloralhydrate (CH), from Merck, interfere in the spindle formation and therefore induce non-disjunctions. Apart from this, FPA is also reported to induce gene mutations and gene conversions (Davies & Parry, 1978), but only in eukaryotic organisms. CH induces (weakly) mutations in Salmonella (Waskell, 1978) and Aspergillus (Bignami et al., 1980).

The fungicide carbendazim (MBC), from BASF, is also known to affect the spindle formation in Aspergillus and thus to induce aneuploids. Also a weak mutagenic action, possibly as a base analogue, has been reported (Kappas, 1981).

The other fungicide tested, vinclozolin (VIN), from BASF, is less investigated but has been reported to induce non-disjunctions in Aspergillus (Vallini et al., 1983) and is a weak inducer of gene mutations in Salmonella and Saccharomyces (Chiesara et al., 1982).

Dimethylsulfoxide (DMSO) has been used to solve MBC, VIN and 9AA (only in part of the experiments). For each experiment, fresh solutions of the mutagens were prepared, except for FPA and ACR. Both chemicals were also used for other purposes (induction of haploids and testing for ACR resistant colonies, respectively) and therefore stock solutions were made, autoclaved and stored at 4 ^{o}C in the dark. These stock solutions were used in testing mutagenicity. The biological activity of these solutions was regularly checked.

2.4 Handling of strains

For normal use, cultures were grown on CM + glu in little flasks for 4 days at 37 °C in an incubator. Those cultures were afterwards stored at 37 °C for longer periods, up to 6 months. Low density conidial solutions were made from these cultures and plated on CM + glu to give single conidium colonies. Conidia from such a single colony were taken and plated on MEA + glu + growth factors and amino acids. In this way it was possible to grow large amounts of conidia with a minimum of mutants or recombinants. After 3 days incubation at 37 °C, the plates were stored at 4 °C prior to making conidial suspensions. If there was doubt of the genetic constitution of the strains kept on flask cultures, new cultures were made starting from conidia stored on dried silica gel. Such a silica gel batch was made by the following procedure: A conidial suspension was centrifugated (10 min, 3000 rpm) and the conidia were resuspended in 5% skimmilk + 5% glutamate. About 0.2 ml from this suspension was added to 0.5 g dried silica gel in little flasks with screw caps, cooled in ice. The silica batches were stored at 4 °C.

2.5 Preparation of conidial suspensions

Conidial suspensions were made by adding saline (8 g NaCl/l distilled water) + tween 80 (0.05 ml/l) to the plates and subsequently suspending the conidia by moving a metallic wire over the surface. The suspensions were heavily shaken for 10 min on a flask shaker and filtered through cotton-wool in order to remove all mycelial debris.

The concentrations of the conidial suspensions were estimated using a Coulter Counter model Z.F. For this purpose dilutions were made to yield a concentration of $4 \times 10^4 - 2 \times 10^5$ conidia/ml.

The conidial suspensions were stored at 4 °C for no longer than 5 days.

2.6 Comparing the conidial size

Conidia of diploid strains are generally larger than those of haploid strains. Therefore the conidial size can be used to distinguish haploid from diploid colonies. For this purpose, the Coulter Counter was employed. By varying the threshold value for the smallest

conidium to be counted, a size distribution of the conidial suspension was obtained. Since such a distribution is characteristic for a haploid or a diploid strain, an unknown colony could be recognized as to be haploid or diploid, since standard strains were included in the test.

2.7 Removal of a germination inhibitor

Scott et al. (1972) reported the existence of a substance on the cell walls of the conidia inhibiting their germination. The normal procedures for preparing conidial suspensions can lead to only partial removal of this inhibitor, resulting in non-reproducible survival data (usually higher numbers) after radiation. An explanation can be that differences in germination time give different opportunities for repair mechanisms. Two procedures for a complete removal of the germination inhibitor have been proposed by Scott and Alderson (1974):

- shaking in a tween 80 solution for 7 hours.
- shaking for 10 min in buffer + 1% diethylether and subsequent removal of the ether by filtering through membrane filters and washing.

In our early experiments, no special care was taken to remove this inhibitor. So it is possible that, during the shaking in tween 80 for only 10 min, not all inhibitor was removed. In the later experiments the ether method, described above, was applied. Whenever this was done, it is stated in the tables.

2.8 Genetic recombination

Sexual and parasexual recombination of the genetic material was used in order to construct new strains, or to analyse recombinants. At first, both in sexual and parasexual recombinations, a heterokaryon was made: conidia of both (haploid) parent strains were mixed together and inoculated on CM + glu. After 24 hours pieces of mycelium were transferred to CM + glu. Growth factors and / or amino acids were added only when both parent strains needed the same growth factor or amino acid and when this was caused by a mutation of the same gene. The transfers were repeated 1 or 2 times, after 3 or 4 days incubation at 37 ^{o}C, until vigorously growing sectors occurred.

2.8.1 Sexual recombination

For sexual recombinations, the heterokaryon was incubated for about 3 weeks at 30 °C until the cleistothecia matured. Some cleistothecia were picked up and rolled over agar plates in order to separate them from the vegetative conidia. Each cleistothecium was crushed and the content was suspended into 0.5 ml saline. It was checked whether the cleistothecia were hybrid or not by streaking a sample on CM + glu and looking for colour markers. The content of one hybrid cleistothecium was used for further analysis. Dilutions were plated on CM + glu to yield 20 - 50 colonies per plate. Then several CM + glu plates were inoculated with 21 of these colonies per plate in such a way that afterwards a template with 21 needles could be used to transfer them to test plates.

2.8.2 Parasexual recombination

For a parasexual recombination, diploid conidia were isolated from a heterokaryon. Therefore the conidia of a fresh heterokaryon were collected and filtered through cotton-wool. These conidia were mixed with ca. 5 ml melted MM + glu (cooled down to 50 °C), and plates were poured. Growth factors and / or amino acids were only added to the MM when the diploid (to be isolated) was expected to need them for growth. After the agar had solidified, a second layer of ca. 15 ml of the same agar was laid on top of the first. After 3 - 4 days incubation at 37 °C, diploid colonies grew through the agar and could be isolated.

When the phenotype of the diploids had been confirmed by growth tests and by estimating the conidial size, conidia were plated in low density to give single cell colonies. Starting from such a single cell colony, this diploid was forced to haploidize by growing on CM + glu + FPA. After 7 days incubation at 37 °C, mycelial ends were transferred to CM + glu. Among the colonies growing on this medium there was a large number of haploids.

3 ANALYSIS OF THE INCUBATION METHODS

Several methods for the incubation of the conidia of the test strains with the chemicals to be tested for mutagenicity were compared. General remarks on these methods are stated in this chapter. The terms mutant / mutation / mutagenicity have been used in a wider sence, including genetic changes other than point mutations.

3.1 Method 1: Plate incorporation assay

The simplest method is to incorporate the chemical to be tested directly into the selection plates, and to inoculate these plates with conidia or mycelium of the test strain. The highest concentration of the chemical to be tested by this method should only give partial inhibition of the growth of Aspergillus. When the conidial colour is to be scored care must be taken that the conidiation is not affected by the mutagen concentration used.

This method is valuable as a pre-screening test, but only qualitative data can readily be obtained. Since we were more interested in quantitative data, method 1 was used only once in this study (see 6.2).

3.2 Method 2: Liquid suspension test

For a more quantitative approach, conidia were treated in a suspension . Such a conidial suspension was made in 0.05 M potassium phosphate buffer, pH 6.8, at a concentration of 10^6 to 10^7 conidia / ml. A sodium citrate buffer, pH 4.5, was only used when nitrite and azide were tested. The chemical to be tested was added to the suspension and this was incubated at 37 °C in a lab shaker, rotating at ca. 250 revolutions per minute, under continuous white light. A 25 ml suspension was shaken in a 100 ml flask and the screw cap was only loosely connected to avoid oxygen shortage. An identical suspension was incubated without the chemical compound (control). After 1 or 2 hours a sample was taken for a viability count on CM + glu + des plates. Then the conidia were separated from the chemical compound by filtering through a membrane filter. The conidia were washed 3 times with sterile

water and afterwards the filter was put in a little flask containing 5 ml saline + tween and was vigorously shaken for 10 min on a mechanical shaker. Appropriate dilutions of the conidial suspension were plated on selective media and on CM + glu + des to measure the mutant frequency.

Using method 2, the comparison of two effects of the mutagen is possible: mutagen frequency and survival. The concentrations of the chemical were chosen in such a way that the survival ranged from about 100% to 10%.

At least 3 independent experiments were performed. The standard deviations were obtained assuming that the numbers of mutants follow binomial distributions and that the errors in estimating the numbers of conidia per plate are relatively small. Katz (1979) gave for a similar situation a formula for the minimal number of plates needed to estimate the conidial number in order to achieve a certain coefficient of variation with a less than 5% risk of stating that a chemical is mutagenic when it is not. This formula is:

$$r \geq 0.41\ (\overline{CV})^2\ (2S)^{1.5}$$

in which r = minimal number of plates for measuring the conidial number

$\overline{CV}$ = approximate coefficient of variation in estimating the number of conidia

S = number of mutants scored

For our experiments, we estimated the value of $\overline{CV}$ from an experiment in which several platings were done from one suspension resulting in a $\overline{CV}$ value of 15%. In our experiments r was usually 3. Thus the total number of mutants scored may not exceed 23, the number calculated from the formula. In order to set a limit to the standard deviation in the binomial distribution in those cases where the mutant numbers exceeded 23, the standard deviation is calculated as if the maximal number was counted and not the actual number. Significance is calculated for $P < 0.05$ (one-sided).

According to Munsun and Goodhead (1977), the enhancement of the mutagenicity frequency should be correlated to the log of the survival rate. Using a linear regression, the slope of the best fit (with a

fixed origin) of each experiment was calculated and these values can be used in comparing the effects of the mutagens.

Since with this method the incubation time is short, only dormant conidia can be tested. Some mutagens, however, act exclusively on dividing cells. Therefore, incubation methods which permit the testing of germinating conidia were developed.

3.3 Method 3: Liquid test with germinating conidia

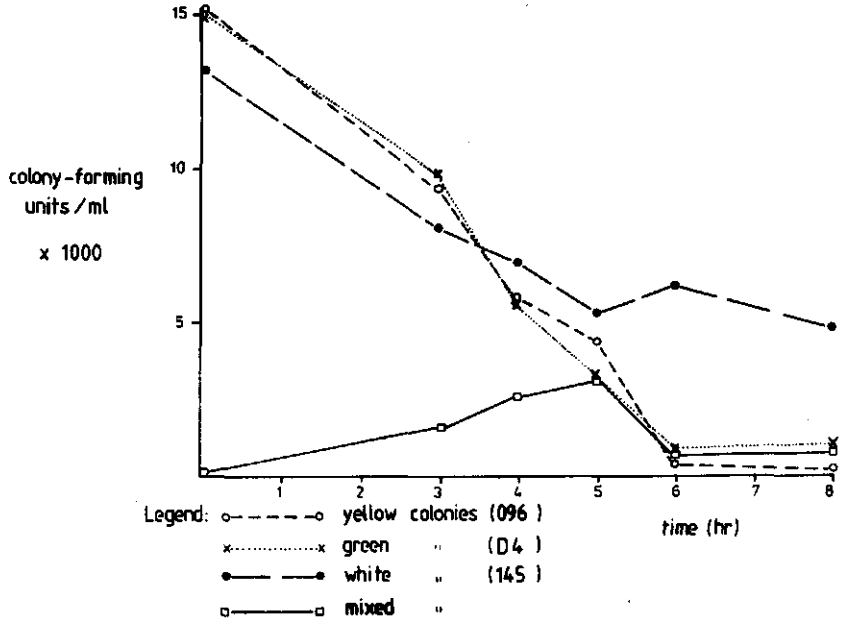
In order to test germinating conidia in liquid suspension, the conidia were suspended in liquid MM, containing only 0.2% agar, supplemented with glucose and the growth factors and amino acids required. This suspension was incubated for 4 hours at 37 °C in a rotating shaker to allow swelling and germination of the conidia without excessive clumping (de Bertoldi et al., 1980). Then the chemical compound was added and the incubation was continued for 1 or 2 hours. A viability count was made by plating on CM + glu + des and the results were compared to the results of the viability count of the mutagen-free control. The mutagen was removed by centrifugation (10 min at 3000 rpm) and the conidia were washed 3 times with sterile water. After being resuspended in saline, these conidia were plated on selective medium as well as on CM.

Although others used this method successfully (de Bertoldi et al., 1980; Gualandi et al., 1979), in the present study method 3 has never yielded very good and reproducible results. Many of the conidia were usually lost at the end of the incubation period in both the control and the mutagen treatment. Microscopic examinations showed that there existed some variation in the degree of germination in the conidial suspension. Most conidia germinated only after 4 - 6 hours incubation but some conidia germinated already at the start, resulting in some big clumps. Removing the germination inhibitor (see chapter 2.7) did not improve the situation.

The following experiment was done to simulate this situation (fig 2): conidia of 3 strains with different conidial colour were mixed together (096 - yellow, 145 - white and D4 - green) in liquid MM + 0.2% agar + glu + paba , pyro , ade, at 37 °C , in a rotating lab shaker,

and at 0, 3, 4, 5, 6 and 8 hours samples were taken and appropriate dilutions were plated on CM + glu + des. These plates were incubated at 37 °C, and after 3 days the number of yellow, white, green and mixed colonies were counted.

Fig 2 Number of colony-forming units found per ml after incubation of a mixture of conidia of the strains 110, 145 and D4 in MM + glu + paba, pyro, ade (0.2% agar, incubation method 3), shaken for several hr at 37 C.



As shown in fig 2, the number of single colour colonies decreased rather dramatically with time whereas the number of mixed colonies increased (initially), even in this low concentrated suspension. There was, however, a considerable difference between the strains. When conidia stick together and give rise to one colony, the mutant frequency measured will not be correct. And when a toxic compound is tested, the clumping of the conidia in the control and in the treatment tube will be different, whereby the survival data will be incorrect, too. Therefore, another method for testing germinating conidia was looked for.

3.4 Method 4: Media mediated assay

The media mediated assay (Bignami et al., 1981) was also used to test germinating conidia: the chemical compound was added to melted CM + glu (cooled down to 50 °C), and plates were poured. These plates were inoculated with a concentrated suspension of conidia of the test strain (ca. 5×10^6 conidia / plate) and incubated for 3 or 4 days at 37 °C. The pH of the medium rises to about 7.5 during growth, even when the initial pH has been adjusted to 4.5 for testing NO2 and N3 activity. Conidial suspensions were made from these plates and the mutant frequency was measured by plating on selective media and on CM + glu + des.

With this method, the chemical compound is present during all (mitotic) cell stages and, if possible, the compound will then be metabolized by the fungus and consequently all metabolites are tested as well.

With this method, a mutant frequency which is not the real mutation frequency is measured, for it is possible that one mutation gives rise to more than one mutant. A mutagenic activity of a compound, of course, will enhance the mutant frequency. But the mutant frequency will also be affected when the mutant mycelium or the conidiation is less inhibited compared to the wildtype (i.e. when a selective advantage exists). Of course, both mechanisms can also act together, or a real mutagenic activity can be obscured by a selective advantage of the wildtypes over the mutants.

In order to distinguish between mutagenic or selective activity, the following control experiments were made: parallel to the normal conidial suspension, the same suspension artificially enriched with mutant conidia was tested. These mutants had been isolated in former experiments with the same tester strain, and usually 21 different isolates were used (inoculated on one plate) in order to simulate the normal genetic variation. If more than one type of mutants (or recombinants) was expected, all these mutants were included in the artificial conidial suspension. The number of mutant conidia in the artificial conidial suspension was usually 10 - 20 times the spontaneous frequency.

If the chemical compound tested has (only) a mutagenic effect on the conidia the mutant frequency in the normal and in the artificial

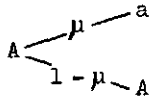
conidial suspension will be equally enhanced. But in the case of a selective advantage the initial mutant frequency of both conidial suspensions will be multiplied with the same factor. So the effect on the artificial conidial suspension will be much greater compared to the effect on the normal suspension.

Model of the situation:

Let a designate the mutant conidia and A the wildtype conidia, and x_1, x_2, x_3 and x_4 the mutant frequencies measured in the 4 different combinations of conidial suspensions and media. p and q are the fractions of wildtype and mutant conidia, respectively, in the artificial conidial suspensions. Let μ be the spontaneous mutant frequency and m the factor by which μ is multiplied as a result of mutagenic activity of the compound, and s the factor by which μ is multiplied due to selective advantage or disadvantage of the mutant conidia in the presence of the compound.

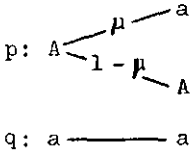
The 4 different situations are:

-1- Normal conidial suspension plated on CM + glu (control)



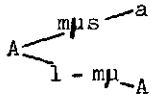
mutant frequency $x_1 = \mu$

-2- Artificial conidial suspension plated on CM + glu



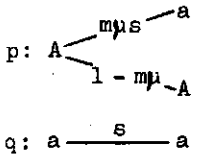
mutant frequency $x_2 = p.\mu + q$

-3- Normal conidial suspension plated on CM + glu + mutagen



mutant frequency $x_3 = \frac{m\mu s}{m\mu s + (1 - m\mu)}$

-4- Artificial conidial suspension plated on CM + glu + mutagen



mutant frequency $x_4 = \frac{pm\mu s + qs}{pm\mu s + qs + p(1 - m\mu)}$

Since μ and q are very small the relations can be simplified to:

$$x_1 = \mu$$
$$x_2 = \mu + q$$
$$x_3 = m\mu s$$
$$x_4 = m\mu s + qs$$

When x_1, x_2, x_3 and x_4 are measured m and s can be calculated using the equations:

$$s = \frac{x_4 - x_3}{x_2 - x_1}$$

$$m = \frac{x_3(x_2 - x_1)}{x_1(x_4 - x_3)}$$

The approximate variance of m and s can be obtained by the method of statistical differentials on the assumption that x_1, x_2, x_3 and x_4 follow Poisson distributions (with the same restriction as in 3.2). However, the shape of the probability distribution is unknown, thus nothing can be concluded about the differences (from 1) being significant. The mutant frequencies of 2 or more plates have been averaged to calculate m and s as if they were estimates of the mutant frequency of the same suspension. In those cases where the differences between the repetitions were found to be significant ($P < 0.05$), this is mentioned in the tables.

4 POINT MUTATION

4.1 General

Several systems for measuring the point mutation frequency in Aspergillus nidulans have been described (see 1.2). In this study the methionine system, originally developed by Lilly (1965), has been used. The advantages of this system over the others are:

- It is in wide-spread use.
- A high frequency is found (in contrast to the arginine system).
- Many conidia can be plated on one plate (in contrast to the 2-thioxanthine system).
- No genetic active compound has to be added to the selection medium (in contrast to the azaguanine system).

A disadvantage, however, is that the methionine prototroph mutants cannot be designated to single loci, in contrast to the 2-thioxanthine system.

In the meth system, prototroph revertants of a strain bearing the methG1 allele were counted. Two strains were available:

470 : proA1 biA1; methG1 frA1 T1(IV;VII); chaA1

110 : biA1; methG1

Compared to strain 470 the sporulation of strain 110 is better, so this strain has been preferred.

After mutagen treatment, the conidia of strain 110 were plated on MM + glu + bio (minimal medium + glucose + biotin) where only revertants can grow. For strain 470, MM + glu + bio, pro (proline) was used. Two conidial concentrations were usually plated: ca. 4×10^5 and 2×10^6 per plate on 5 plates each. Whenever a very high mutant frequency was expected, lower concentrations were used. The plates were incubated for 4 days at 37 °C. The actual number of conidia was estimated by plating an appropriate dilution on CM + glu + des (complete medium + glucose + desoxycholate), and these plates were incubated for 2 days at 37 °C.

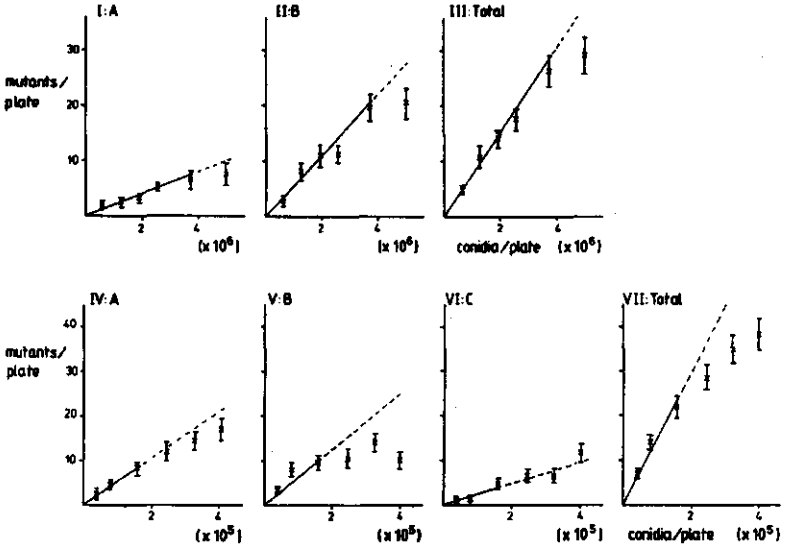
The methionine prototroph revertants can be assigned to 3 classes with different morphology. Class A mutants have wildtype-like colonies. Class B colonies are aconidiate and their mycelium excretes a brownish

pigment into the agar. The colonies of class C mutants are densely conidiated in the centre and have a hyaline edge.

Lilly investigated several mutants of all 3 classes by sexual analysis and she found out that both class A and C contained mutants at at least 2 different loci, which segregate independently from each other and from the original methG1 locus. Mutants of class B could not be analysed since crosses between type B mutants were sterile. Scott and Alderson (1971) gave indirect evidence that 2 loci exist for class B mutations. They observed in mutation experiments with NUV-light (near ultra-violet) and 8-methoxypsoralen that there was a correlation between mutant yield and the number of loci involved. Applied to the meth system, mutants at all 3 classes were about equally induced. Therefore, they concluded that class B will also probably contain mutants at 2 loci.

Because of the different growth characteristics, the type A mutants (and the type C, too, but there are usually less of them) easily overgrow the type B mutants. Therefore, the number of mutants per plate may not be too high. Fig 3 shows the number of mutants scored by us in

Fig 3 Mutants per plate found after inoculation of different concentrations of untreated and MMS-treated (17 mM, method 2) conidial suspensions of strain 110 on MM + glu + bio. I: type A mutants/control, II: B/control, III: total/control, IV: A/MMS, V: B/MMS, VI: C/MMS and VII: total/MMS (C/control is not shown since there were only few type C mutants). The mutant frequency, shown as a line, is calculated from the total results of the plates with less than 25 colonies per plate.



Legend: x average number of mutants per plate.

a series of dilutions of one conidial suspension plated on selective medium. Up to a mutant concentration of ca. 25 colonies per plate, no significant differences between the calculated mutant frequencies have been found. At higher concentrations the type B mutants are underestimated.

4.2 Results

The results obtained with strain 110 (and in one case with strain 470) are shown in the tables 2 - 17. In applying incubation method 2 (tables 2 - 8), as a rule more than one independent experiment has been performed, and in those experiments more than one concentration of the compound was usually tested. At each concentration the survival rate (given as percentage of the control) was measured, the number of revertants belonging to each class was counted and the revertant rate was calculated. The standard deviation (for estimation see 3.2) and the significances of the results were only calculated for the revertant rate of all classes combined. The mutagen concentrations have been chosen in such a way that, when possible, at the highest concentrations there was a considerable impact on the survival.

In order to be able to compare the effects of the different compounds, the enhancement of the mutant frequency over the control at different mutagen concentrations within the same independent experiment have been correlated to the natural logarithm of the survival rate. The slope of the regression line $y = ax$ indicates the importance of the mutagenic effects in relation to the cell killing properties of the compound. A fixed origin was chosen since the errors in measuring the spontaneous mutant frequency will be much smaller than the errors of the mutant frequencies at a (high) mutagen concentration.

In applying incubation method 4 (tables 10 - 17), the toxic effects of a compound can only be indicated by scoring the conidiation of the test strain in the presence of the compound, and this score is given in the tables. The concentrations of the compound have been chosen in such a way that, at the highest concentrations, there was an evident influence on the conidial colour. In those cases where also an artificial conidial suspension has been tested, the mutagenicity factor (m) and the selection factor (s) has been calculated according to chapter 3.4.

Table 2 Mutant frequencies (mutants per 10^5 survivors) after treatment of conidia of strain 110 with methylmethanesulfonate for 1 hr at 37 °C in liquid suspension, pH 6.8 (incubation method 2). In parenthesis the mutant numbers are shown.

exp.	conc. (mM)	surv. %	class: A		B		C		total		s.d.	regression y = ax
1 rem	0	100	(7)	0.08	(70)	0.82	(8)	0.09	(85)	1.0	0.21	
	17	82	(16)	3.5	(47)	10	(16)	3.5	(79)	17 *	3.6	
	23	54	(33)	8.7	(48)	13	(10)	2.6	(91)	24 *	4.9	
	23	49	(21)	4.8	(76)	17	(26)	6.0	(123)	28 *	5.8	a = -31
	28	56	(21)	6.9	(34)	11	(17)	5.5	(72)	24 *	4.8	r = -0.98
	33	19	(24)	8.8	(77)	28	(20)	7.3	(121)	44 *	9.1	
	33	15	(13)	26	(15)	30	(3)	6	(31)	63 *	13	
2 rem	0	100	(12)	0.16	(48)	0.64	(7)	0.09	(67)	0.91	0.19	
	23	83	(20)	15	(32)	24	(10)	7.5	(62)	47 *	9.6	a = -208
	28	87	(34)	8.9	(67)	17	(18)	4.7	(119)	31 *	6.4	r = -0.98
	33	76	(30)	16	(55)	30	(10)	5.5	(95)	52 *	11	
3 nrem	0	100	(13)	0.10	(55)	0.41	(8)	0.06	(76)	0.57	0.12	
	17	48	(71)	5.8	(17)	1.4	(53)	4.3	(141)	11 *	2.4	a = -27
	23	13	(56)	18	(20)	6.6	(36)	12	(112)	37 *	7.6	r = -0.91
	27	19	(57)	34	(32)	19	(29)	17	(118)	71 *	15	

Legend to tables 2 - 9:

rem	germination inhibitor removed
nrem	germination inhibitor not removed
A, B, C	mutant classes according to Lilly (1965)
s.d.	standard deviation (only given for all classes combined)
*	significantly different from the control value ($P < 0.05$, one-sided)
a	slope of the regression y = ax, in which y represents the induced mutation frequency diminished with the control value, and x is ln(survival rate)
r	correlation coefficient

Table 3 Mutant frequencies (mutants per 10^5 survivors) after treatment of conidia of strain 110 with sodium azide for 1 hr at 37 °C in liquid suspension, pH 4.5 (incubation method 2). In parenthesis the mutant numbers are shown.

exp.	conc. (mM)	surv. %	class: A		B		C		total		s.d.	regression y = ax
1 rem	0	100	(10)	0.07	(45)	0.33	(1)	0.009	(56)	0.41	0.08	
	15	76	(19)	0.15	(27)	0.21	(3)	0.02	(49)	0.39	0.08	
	31	81	(13)	0.06	(33)	0.16	(1)	0.005	(47)	0.23	0.05	a = -2.7
	31	74	(19)	0.16	(38)	0.33	(2)	0.02	(59)	0.51	0.10	r = -0.62
	77	71	(15)	0.88	(34)	2.0	(0)		(49)	2.9 *	0.6	
2 rem	0	100	(8)	0.16	(27)	0.54	(4)	0.08	(39)	0.77	0.16	
	116	8	(13)	33	(7)	18	(8)	20	(28)	70 *	14	a = -27
3 rem	0	100	(12)	0.18	(18)	0.27	(3)	0.04	(33)	0.49	0.10	
	37	71	(47)	1.0	(18)	0.37	(1)	0.02	(66)	1.4 *	0.3	
	70	59	(36)	4.7	(31)	4.1	(1)	0.13	(68)	8.9 *	1.8	
	110	51	(74)	11	(39)	5.9	(7)	1.1	(120)	18 *	3.8	a = -20
	110	15	(52)	26	(13)	6.5	(1)	0.5	(66)	33 *	6.6	r = -0.99
	146	2.1	(94)	72	(9)	7	(1)	0.7	(104)	79 *	16	

Legend: see table 2

Table 4 Identical situation as in table 3 except for the pH: here sodium azide is tested at pH 6.8.

experiment	conc. (mM)	surv. %	class: A		B		C		total		s.d.
1 rem	0	100	(25)	0.12	(40)	0.19	(6)	0.03	(71)	0.33	0.07
	77	92	(16)	0.09	(39)	0.21	(7)	0.04	(62)	0.33	0.07
	308	79	(12)	0.10	(40)	0.35	(11)	0.09	(63)	0.55	0.11

Legend: see table 2

Table 5 Mutant frequencies (mutants per 10^5 survivors) after treatment of conidia of strains 110 and 470 with butadienediepoxide for 1 hr at 37 °C in liquid suspension, pH 6.8 (incubation method 2). In parenthesis the mutant numbers are shown.

exp.	conc. (mM)	surv. %	class: A		B		C		total		s.d.	regression y = ax
1 rem	0	100							(59)	0.77	0.16	
(470)	6.4	73							(178)	15 *	3	a = -110
	19.4	34							(85)	126 *	26	r = -0.986
2 nrem	0	100							(87)	0.36	0.07	
(470)	6.4	73							(61)	2.4*	0.5	a = -100
	19.4	42							(42)	98 *	20	r = -0.95
3 rem	0	100	(70)	0.20	(183)	0.53	(10)	0.03	(263)	0.77	0.16	
(110)	6.4	78	(62)	5.2	(8)	7.4	(28)	2.4	(178)	15 *	3	a = -177
	19.4	33	(66)	132	(35)	70	(1)	2	(102)	204 *	42	r = -0.989

Legend: see table 2

Table 6 Mutant frequencies (mutants per 10^5 survivors) after treatment of conidia of strain 110 with 9-aminoacridine, solved in DMSO, for 1 or 2 hr at 37 °C in liquid suspension, pH 6.8 (incubation method 2). In parenthesis the mutant numbers are shown.

experiment	conc. (mM)	surv. %	class: A		B		C		total		s.d.
1 nrem	0	100	(9)	0.06	(13)	0.09	(3)	0.02	(25)	0.16	0.03
(1 hr)	0.16	100	(10)	0.10	(12)	0.12	(2)	0.02	(24)	0.23	0.05
	0.32	100	(10)	0.08	(12)	0.09	(2)	0.02	(24)	0.18	0.04
	0.64	72	(12)	0.10	(14)	0.12	(2)	0.02	(28)	0.24	0.05
2 nrem	0	100	(21)	0.10	(84)	0.41	(18)	0.09	(123)	0.60	0.12
(1 hr)	0.31	100	(28)	0.15	(83)	0.45	(12)	0.06	(123)	0.66	0.14
	0.62	100	(19)	0.14	(68)	0.49	(16)	0.12	(103)	0.74	0.15
3 nrem	0	100	(10)	0.10	(60)	0.60	(10)	0.10	(80)	0.79	0.16
(2 hr)	0.29	100	(13)	0.20	(50)	0.77	(5)	0.08	(68)	1.04	0.21
	0.39	100	(17)	0.11	(51)	0.34	(9)	0.06	(77)	0.52	0.11

Legend: see table 2

Table 7 Mutant frequencies (mutants per 10^5 survivors) after treatment of conidia of strain 110 with acriflavine for 2 hr at 37 °C in liquid suspension, pH 6.8 (incubation method 2). In parenthesis the mutant numbers are shown.

exp.	conc. (mM)	surv. %	class: A		B		C		total		s.d.	regression y = ax
1 nrem	0	100	(15)	0.16	(42)	0.46	(2)	0.02	(59)	0.65	0.13	
	0.073	66	(32)	0.49	(68)	1.0	(4)	0.06	(104)	1.6 *	0.3	a = -1.41
	0.15	44	(28)	0.46	(78)	1.3	(7)	0.11	(113)	1.9 *	0.4	r = -0.988
	0.29	21	(24)	1.1	(31)	1.4	(5)	0.23	(60)	2.7 *	0.6	
2 nrem	0	100	(2)	0.08	(23)	0.90	(3)	0.12	(28)	1.1	0.2	
	0.15	89	(14)	0.76	(29)	1.6	(8)	0.43	(51)	2.8 *	0.6	
	0.29	13	(24)	0.64	(59)	1.6	(16)	0.43	(99)	2.6 *	0.5	a = -0.73
	0.44	4	(13)	1.0	(45)	3.4	(6)	0.46	(64)	4.9 *	0.1	r = -0.89
	0.58	0.75	(5)	1.4	(8)	2.3	(0)		(13)	3.7 *	1.0	
3 rem	0	100	(12)	0.12	(32)	0.31	(11)	0.11	(55)	0.54	0.11	
	0.21	18	(12)	0.87	(19)	1.4	(5)	0.36	(36)	2.6 *	0.5	a = -1.21
	0.43	43	(19)	0.77	(17)	0.69	(4)	0.16	(40)	1.6 *	0.3	r = -0.9998
4 rem	0	100	(18)	0.11	(62)	0.39	(8)	0.05	(88)	0.55	0.11	
	0.44	7	(15)	1.8	(34)	4.1	(3)	0.36	(52)	6.3 *	1.3	a = -2.2
	0.44	9	(14)	1.4	(44)	4.6	(1)	0.1	(59)	6.1 *	1.3	r = -0.9995

Legend: see table 2

Table 8 Mutant frequencies (mutants per 10^5 survivors) after treatment of conidia of strain 110 with sodium nitrite for 1 hr at 37 °C in liquid suspension, pH 4.5 (incubation method 2). In parenthesis the mutant numbers are shown.

exp.	conc. (mM)	surv. %	class: A		B		C		total		s.d.	regression y = ax
1 nrem	0	100	(132)	0.64	(23)	0.11	(3)	0.01	(158)	0.77	0.16	
	33	10	(34)	37	(9)	9.9	(23)	25	(66)	72 *	15	a = -79
2 nrem	0	100	(62)	0.85	(18)	0.25	(2)	0.03	(82)	1.1	0.2	
	13.5	55	(69)	1.4	(28)	0.56	(34)	0.67	(131)	2.6*	0.5	a = -3.3
3 nrem	0	100	(14)	0.10	(19)	0.13	(0)		(33)	0.23	0.05	
	20	48	(42)	5.3	(6)	0.8	(26)	3.3	(74)	9.3*	1.9	
	20	32	(35)	5.7	(4)	0.7	(32)	5.2	(71)	11 *	2	a = -13.4
	33	8	(60)	22	(4)	1.5	(48)	18	(112)	41 *	8	r = -0.982
	33	8.5	(67)	15	(4)	0.9	(51)	12	(122)	28 *	6	
4 nrem	0	94	(28)	0.08	(21)	0.06	(19)	0.06	(68)	0.20	0.04	
	0	107	(28)	0.13	(39)	0.18	(15)	0.07	(82)	0.38	0.08	
	20	97	(96)	2.9	(11)	0.33	(58)	1.8	(165)	5.0*	1.0	a = -12.6
	30	51	(112)	5.5	(9)	0.44	(79)	3.9	(200)	9.9*	2.0	r = -0.96
	30	34	(97)	7.1	(10)	0.73	(77)	5.6	(184)	13 *	3	

Legend: see table 2

Table 9 Mutant frequencies (mutants per 10^5 survivors) after treatment of pre-germinated conidia of strain 110 with 9-aminoacridine for 2 hr at 37 °C in liquid medium at pH 6.8 (incubation method 3). In parenthesis the mutant numbers are shown.

experiment	conc. (mM)	surv. %	class: A		B		C		total		s.d.
1 rem	0	100	(14)	0.40	(7)	0.20	(2)	0.06	(23)	0.65	0.14
in 1.6%	0.016	97	(13)	0.36	(11)	0.30	(1)	0.03	(25)	0.69	0.14
DMSO	0.16	90	(12)	0.37	(10)	0.31	(0)		(22)	0.68	0.15
	1.6	100	(5)	0.28	(6)	0.34	(0)		(11)	0.63	0.19
2 rem	0	100	(32)	0.58	(3)	0.05	(3)	0.05	(38)	0.69	0.14
in 0.4%	0.35	100	(55)	0.79	(3)	0.04	(1)	0.01	(59)	0.85	0.18
DMSO	0.88	100	(21)	0.24	(4)	0.05	(0)		(25)	0.28	0.06

Legend: see table 2

Table 10 Total mutant frequencies (mutants per 10^5 conidia) after growth of strain 110 on CM + glu butadienediepoxide (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with mutants were tested. Incubation for 3 days at 37 °C.

			110			110 + enrichment				
exp.	conc. (mM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	40	3.0 e	0.6	68	4.6	1.0		
			127	1.5 e	0.3	24	5.2	1.1		
			19	1.1 e	0.3	87	3.8	0.8		
	0.13	+	29	8.0 *e	1.6	39	11	2		
			28	3.0 e	0.6	37	13	3	m = 2.5	1.6
			32	11 *e	2	32	10	2	s = 1.6	0.8
	0.26	+	40	14 *	3	45	13	3		
			47	11 *	2	45	14	3	m =34	173
			50	14 *	3	41	13	3	s = 0.2	1.0
	0.52	(-)	140	67 *	13	125	37	8		
			55	64 *	13	21	54	11	g	
			51	80 *	17	225	81	17		

legend:
+ dark-green conidia
(+) light-green conidia
(-) conidia only faintly coloured
- no conidial colour seen
m multiplying effect on the spontaneous mutant frequency caused by mutagenicity
s multiplying effect on the spontaneous mutant frequency caused by selective advantage
e the difference between the repetitions is significant ($P<0.05$, two-sided)
s.d. standard deviation
n.s. not scored
* significantly greater than the highest mutant frequency of the controls ($P<0.05$, one-sided)
g m and s are negative since the mixture yielded less mutants than the original suspension

Table 11 Total mutant frequencies (mutants per 10^5 conidia) after growth of strain 110 on CM + glu acriflavine (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with mutants were tested. Incubation for 3 days at 37 °C.

			110			110 + enrichment				
exp.	conc. (µM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	40	3.0 e	0.6	68	4.6	1.0		
			127	1.5 e	0.3	24	5.2	1.1		
			19	1.1 e	0.3	87	3.8	0.8		
	9	(+)	62	0.87	0.18	77	5.7 e	1.2		
			123	1.8	0.4	80	5.6 e	1.2	m=0.53	0.23
			23	1.0	0.2	60	2.4 e	0.5	s=1.3	0.4
	23	(+)	35	1.3 e	0.3	58	5.0	1.0		
			11	0.34e	0.10	38	2.7	0.6	m=0.39	0.18
			21	0.91e	0.20				s=1.1	0.38
2	0	+	100	1.5	0.4	123	72	17		
			39	2.7	0.6	96	60	14		
	23	(-)	7	0.7		67	95	20	m=0.34	0.15
			6	0.8		17	42	10	s=1.1	0.29
	32	(-)	5	2.5		38	211	50	m=0.36	0.21
									s=3.3	1.0
3	0	+	22	3.5	0.8	68	45	9		
			21	2.3	0.5	54	44	9		
	28	(-)	206	5.0	0.4	205	142	29	m=0.6	0.2
						152	119	25	s=3	0.7

Legend: see table 10

Table 12 Total mutant frequencies (mutants per 10^5 conidia) after growth of strain 110 on CM + glu + sodium nitrite (incubation method 4). The pH of the agar was adjusted to 4.5. Both normal conidial suspensions and suspensions artificially enriched with mutants were tested. Incubation for 3 or 4 days at 37 °C.

			110			110 + enrichment				
exp.	conc. (mM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	66	6.7	1.4	73	32	7		
(4 days)			66	5.9	1.2	73	35	7		
			60	8.3	1.7	51	30	6		
	0.85	+	78	6.0	1.2	24	14	3		
			60	5.8	1.2	58	27	6	m=1.8	0.7
			61	7.2	1.5	26	17	4	s=0.5	0.15
	1.8	(+)	92	7.4	1.5	221	127	26		
			96	11	2	160	78	16	m=0.4	0.1
			85	12	3	255	120	25	s=3.9	1.0
2	0	+	30	4.3	1.0	90	7.9	1.9		
(3 days)			115	2.3	0.5	64	7.7	1.8		
	0.46	+	44	3.0	0.7	90	6.8	1.6	m=2.5	2.2
			43	6.3	1.5	68	7.6	1.8	s=0.6	0.4
	0.92	+	36	3.9	0.9	92	5.8	1.4	m=1.1	0.7
			43	1.9	0.5	80	7.7	1.7	s=0.9	0.4
	1.8	-	46	4.2	1.0	44	19	5	m=0.5	0.3
			62	6.1	1.4	25	17	4	s=2.9	1.3
3	0	+	34	4.0	0.8	79	48	10		
(3 days)			31	4.2	0.9	48	38	8		
	1.38	+	44	6.5	1.3	66	43	9	m=1.9	0.7
			52	6.1	1.3	64	32	7	s=0.8	0.2
	1.7	(+)	26	3.0	0.6	284	206	42	m=0.1	0.03
			23	2.1	0.4	438	309	64	s=6.5	1.6

Legend: see table 10

Table 13 Total mutant frequencies (mutants per 10^5 conidia) after growth of strain 110 on CM + glu + sodium azide (incubation method 4). The pH of the agar was adjusted to 4.5. Both normal conidial suspensions and suspensions artificially enriched with mutants were tested. Incubation for 3 or 4 days at 37 °C.

			110			110 + enrichment				
exp.	conc. (µM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	66	6.7	1.4	73	32	7		
(4 days)			66	5.9	1.2	73	35	7		
			60	8.3	1.7	51	30	6		
	38	+	56	5.0	1.0	32	24	5		
			72	6.0	1.2	35	18	4	m=1.35	0.5
			44	4.8	1.0	30	16	3	s=0.56	0.15
	76	+	82	7.1	1.5	62	49	10		
			70	6.2	1.3	53	21	4	m=0.79	0.28
			147	3.7	0.8	49	25	5	s=1.0	0.26
2	0	+	34	4.0	0.8	79	48	10		
(3 days)			31	4.2	0.9	48	38	8		
	48	(+)	69	1.5 e	0.3	268	31	6	m=1.9	0.7
			61	7.3 e	1.5	126	22	5	s=0.6	0.15
	60	(-)	28	3.4	0.7	60	31	6	m=1.2	0.5
						36	30	6	s=0.7	0.2
3	0	+	30	4.3	1.0	90	7.9	1.9		
(3 days)			115	2.3	0.5	64	7.7	1.8		
	14	+	98	1.8	0.4	15	3.9	0.9	m=0.9	0.6
			71	1.1	0.3	43	3.2	0.8	s=0.5	0.23
	28	(+)	85	1.1	0.3	71	3.2	0.8	m=0.35	0.23
						101	7.5	1.8	s=0.9	0.5

Legend: see table 10

Table 14 Total mutant frequencies (mutants per 10^5 conidia) after growth of strain 110 on CM + glu methylmethanesulfonate (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with mutants were tested. Incubation for 3 days at 37 °C.

			110			110 + enrichment				
exp.	conc. (mM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	129	1.2	0.2					
			111	0.93	0.19					
	0.59	(+)	92	5.2 *	1.1					
			102	4.3 *	0.9					
	1.76	(-)	309	18 *	4					
			439	26 *	5					
2	0	+	85	0.93	0.19					
			61	0.74	0.15					
			84	0.71	0.15					
	0.29	(+)	175	1.0	0.2					
			151	1.5	0.3					
	0.59	(+)	77	7.1 *	1.5					
			69	6.8 *	1.4					
			77	9.0 *	1.9					
	1.17	(+)	116	15 *	3					
			117	16 *	3					
3	0	+	126	1.8	0.4	127	22	5		
			79	1.4	0.3	138	29	6		
			120	1.7	0.4	16	23	6		
	0.29	+	18	17 *	4	38	53	11		
			13	12 *	3	75	74	15	m= 3.1	1.1
			106	9.3 *	1.9	57	80	16	s= 2.5	0.6
	0.59	(+)	38	38 *	8	113	144	30		
			31	32 *	7	126	72	15	m= 6.1	2.3
						209	131	27	s= 3.5	0.9
	1.17	(-)	89	425 *	88	126	484	100		
			90	375 *	77	141	526	108	m=16.6	8.1
			67	209 *	43	103	858	177	s=12.4	4.8

Legend: see table 10

Table 2 shows the results of 3 experiments in which methylmethanesulfonate / method 2 was tested. A large effect on the revertant frequencies of all 3 classes has always been found. In the second experiment, however, the survival was found to be much higher than in the 2 others, resulting in a much smaller slope of the regression line. Applying method 4 (table 14), MMS was found to increase the revertant number strongly. In 1 experiment an artificial suspension has also been tested, and both m and s were found to be increased.

Butadienediepoxide was also found to be a very active mutagen. Using method 2, at the highest concentrations BED predominantly class A and B mutants were induced (table 5). With method 4, only 1 experiment has been performed (table 10), in which, unfortunately, too low numbers of revertants had been added to the artificial suspension. So m and s could not be measured accurately. It can, however, be expected that there is only an effect on m, since the difference between the results

Table 15 Total mutant frequencies (mutants per 10^5 conidia) after growth of strain 110 on CM + glu 9-aminoacridine (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with mutants were tested. Incubation for 3 days at 37 °C.

			110			110 + enrichment				
exp.	conc. (mM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	125	0.72	0.15					
in 0.2%			115	1.1	0.2					
DMSO	0.14	+	66	0.61	0.13					
			106	1.0	0.2					
	1.4	+	74	0.78	0.16					
			126	1.7	0.4					
2	0	+	53	0.57	0.12					
in 0.2%			89	0.84	0.17					
DMSO	0.77	+	78	0.60	0.12					
			126	0.94	0.19					
	1.5	+	97	0.84	0.17					
			142	0.87	0.18					
3	0	+	48	4.0	0.9	113	40	9		
in H_2O						58	21	5		
	0.13	+	362	5.0	1.2	307	27	6	m= 1.3	0.7
			244	4.1	1.0				s= 0.9	0.3
	0.26	(+)	33	13 *	3	156	24	6	m= 2.8	1.4
			23	11 *	3	174	59	14	s= 1.1	0.4
4	0	+	126	1.8	0.4	127	22	5		
in H_2O			79	1.4	0.3	138	29	6		
			120	1.7	0.4	16	23	6		
	0.24	(+)	21	6.9*	1.5	70	29	6		
			27	9.2*	1.9	74	30	6	m= 5.1	1.8
			126	10 *	2	66	40	8	s= 1.1	0.3
	0.47	(+)	80	26 *	5	180	82	17		
			27	14 *	3	45	33	7	m= 7.5	3.0
			70	20 *	4				s= 1.6	0.5
	0.97	(+)	10	23 *	7	28	42	9	m=13.9	12.9
			3	7.8		12	20	6	s= 0.7	0.4

Legend: see table 10

of the two suspensions was about constant at all BED concentrations tested.

Sodium nitrite was very active at pH 4.5 when method 2 was applied. Predominantly, class A and C revertants were found to be induced. Using method 4 no mutagenicity was found but the revertants were shown to have a selective advantage in the presence of nitrite (tables 8 and 12).

Sodium azide (method 2) was found to be mutagenic at pH 4.5, although there was a considerable difference between the results of the 3 experiments shown in table 3. In the first experiment lower N3 concentrations were tested, resulting in much lower revertant numbers. Besides, the distribution of the revertants over the 3 classes was different in the 3 experiments. At pH 6.8, even at higher N3 concentrations (table 4), no mutagenic activity was found. With method 4 (pH 4.5), no mutagenicity was found (table 13).

Table 16 Total mutant frequencies (mutants per 10^5 conidia) after growth of strain 110 on CM + glu carbendazim (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with mutants were tested. Incubation for 3 days at 37 °C.

			110			110 + enrichment				
exp.	conc. (µM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	n.s.	51	0.89	0.18					
in 0.1%			29	0.53	0.11					
DMSO	0.5	n.s.	30	1.4	0.3					
			28	0.79	0.16					
	1.0	n.s.	43	1.3	0.3					
			45	1.0	0.2					
	1.6	n.s.	45	0.84	0.17					
			57	0.84	0.17					
2	0	+	77	1.0	0.1					
in 0.5%			81	1.1	0.23					
DMSO			67	0.75	0.15					
	1.3	+	73	0.74	0.15					
			91	0.76	0.16					
	2.6	-	25	0.74 e	0.15					
			29	0.61 e	0.13					
			89	2.7 *e	0.6					
3	0	+	151	2.6	0.6	46	68	16		
in 0.4%			42	4.5	1.1	35	54	13		
DMSO	0.65	+	39	5.8	1.4	59	60	14	m=1.5	0.6
			41	3.5	0.8	45	51	12	s=0.9	0.3
	0.98	+	33	3.8	0.9	44	68	16	m=0.9	0.4
			23	2.1	0.5	34	44	10	s=0.9	0.4
	1.51	(-)	56	1.2	0.3	48	9.6	2.3	m=1.7	0.7
			77	0.47	0.11	53	8.7	2.0	s=0.14	0.04
	1.83	(-)	41	0.88	0.21	74	20	5	m=1.2	0.5
			66	1.9	0.5	24	22	5	s=0.34	0.1

Legend: see table 10

Table 17 Total mutant frequencies (mutants per 10^5 conidia) after growth of strain 110 on CM + glu vinclozolin (incubation method 4). Only normal suspension were tested. Incubation for 3 days at 37 °C.

exp.	conc. (µM)	coni-diation	no.	freq.	s.d.
1	0	+	53	0.57	0.12
in 0.2%			89	0.84	0.17
DMSO	0.87	+	99	0.83	0.17
			59	0.45	0.09
	1.7	+	76	0.65	0.13
	3.5	+	65	0.69e	0.14
			44	0.25e	0.05
	7.0	(+)	145	1.0	0.21
			90	0.79	0.16
2	0	+	70	0.46	0.09
in 0.2%			66	0.44	0.09
DMSO			39	0.27	0.06
	1.7	+	71	0.50e	0.10
			54	0.30e	0.06
			16	0.16e	0.04
	3.5	+	90	0.78	0.16
			64	0.57	0.12
			76	0.56	0.12
	7.0	(+)	108	0.73	0.15
			70	0.46	0.10
			96	0.77	0.16

Legend: see table 10

Acriflavine was found to be mutagenic when tested with method 2 (two hours incubation time), but the effect was small and only found at a very low survival rate (table 7), resulting in a slope of the regression line which was much nearer to zero compared to the results with the compounds discussed before. Only class A and B revertants were unambiguously found to be induced. Method 4 did not reveal a mutagenic activity. The only effect was that in the presence of ACR the mutants had some selective advantage over the wildtypes (table 11).

9-Aminoacridine, solved in DMSO, was not found to be mutagenic in a concentration up to 0.65 mM, both with methods 2 and 3 (tables 6 and 9), and little or no effect on the survival rate was observed. The results with method 4 depended on the solvent used: solved in DMSO, no mutagenic activity was seen, but when 9AA was directly solved in warm agar, a clear mutagenic effect was found and m was greater than 1 (table 15).

Carbendazim was only tested using method 4, and no reproducible increase in the mutant frequency was observed (table 16). In analysing the m and s factors in one experiment, m was always about 1 or slightly higher, but s became much smaller than 1 at the highest concentrations.

Vinclozolin, too, was only tested using method 4, and no influence on the mutant frequency was found (table 17).

4.3 Preliminary conclusions

The methionine system is suitable for testing mutagenic properties. Separating different classes (A, B and C) is possible and shown for the experiments with method 2.

MMS and BED are both potent inducers of point mutations. The variation in the results of the repeated experiments prevents an exact comparison of the effects of those two mutagens.

NO2 and N3 are both only active at pH 4.5 and only with method 2. They are less active than MMS and BED.

ACR is only weakly mutagenic and, here too, the only effect was obtained with method 2.

9AA never gives positive results when solved in DMSO. However, without this solvent and using method 4, a clear mutagenic activity is shown.

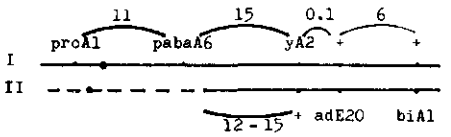
MBC and Vin are not shown to be mutagenic (only tested with method 4).

5 LOSS OF A DUPLICATION FRAGMENT

5.1 General

Another genetic end-point which can be measured in Aspergillus nidulans is the instability of duplication strains during mitosis. In a duplication strain, part of a chromosome is present twice in the otherwise haploid genome. One copy is in normal position and the other copy is present as a translocation connected to another chromosome. All duplication strains found until now can be distinguished from normal strains because of the typical morphology of their colonies and their reduced growth rate. This phenomenon is called "crinkled" (Bainbridge & Roper, 1966). In crinkled colonies sometimes sectors with a wildtype morphology spontaneously arise. But also sectors with a morphology which is intermediate between the crinkled and the wildtype morphology and even sectors with a deteriorated morphology have been observed (Nga & Roper, 1968). Chemicals like coumarin and caffeine, and UV radiation enhanced the instability of duplication strains (Majerfeld & Roper, 1978; Roper et al., 1972; Normansell & Holt, 1979). The wildtype morphology reappears when the translocated fragment or the fragment in normal position is deleted and a balanced genome is regained. The presence of a duplication fragment induces its own instability, but the mechanism involved is not known.

In this study the I→II duplication, originally isolated by Pritchard (1960), has been used. The sectors arising from this duplication strain most frequently lost the translocated fragment, and not the fragment in normal position (Lieber, 1976). The genotype of strain 002 (obtained from prof. Roper, Sheffield) is:



The meiotic map distances are by Clutterbuck (1981). The transformation break-point was calculated to be immediately distal to the pabaA6 locus (van de Vate & Jansen, 1978).

5.2 Handling of strain 002

After mutagen treatment, conidia of strain 002 were plated on CM + glu to yield about 300 colonies per plate. Incubation was for 4 days at 37 °C, and the number of -independent- yellow sectors were counted. A differentiation was made between sectors arising early ($\frac{1}{4}$ or more of the colony was yellow) and sectors arising late (less than $\frac{1}{4}$ of the colony was yellow) in colony development. Table 18 gives the accumulated results of several experiments in which conidia of strain 002 were treated with 17.7 mM MMS using incubation method 2.

Table 18 Number and frequency (per 10^4 colonies) of sectors arising after plating on CM + glu of conidia of strain 002. Treatment was with 17.7 mM MMS in liquid suspension, pH 6.8, for 1 hour at 37°C (method 2). Accumulated results of several experiments.

	control		MMS treated	
	no.	freq.	no.	freq.
early sectors	7	1.9	37	9
late sectors	22	4.6	52	12.5

5.3 Construction of a duplication strain carrying the sorA2 allele

In order to obtain another selective advantage of the yellow sectors over the parental colonies and to be able to inoculate more colonies per plate, a duplication strain carrying one copy of the sorA allele (resistance to sorbose) was constructed. SorA was known to be localized on the right arm of chromosome I (Elorza & Arst, 1971; and own investigations, data not shown). Out of cross 003 x 002 several strains were isolated which - just like strain 002 - had a crinkled morphology, needed the supplementation of proline (pro) and para-aminobenzoic acid (paba) for growth on MM and were sensitive to sorbose. These strains were treated with MMS and one of them, strain 007, yielded yellow sectors which were sorbose resistant and adenine (ade) and biotin (bio) prototroph. The genotype of strain 007 was investigated by genetic analysis of this strain and its sectors:

- Strain 007 was "selfed": a colony was grown on CM + glu and after 3 weeks incubation at 37 °C, some (small) cleistothecia were crushed and the content plated on CM + glu in appropriate dilutions. Among the 200 descendents there were yellow, adenine auxotroph, biotin auxotroph and / or sorbose resistant colonies (table 19).

Table 19 Selfing of strain 007. The offspring was grown on MM + ace + pro, paba, ade, bio. Adenine and biotin prototrophs were scored as not growing when ade or bio respectively were omitted. Sorbose resistance was scored on medium supplemented with sorbose.

phenotypes among the progeny

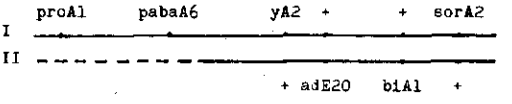
y	ad	bi	sor	no.
+	+	+	+	144
+	+	+	-	18
+	+	-	+	8
+	+	-	-	2
+	-	-	+	12
y	+	+	+	9
y	+	+	-	7
				200

Legend: + wildtype growth or colour
- mutant growth
y yellow colour

Other phenotypes were not found.

- The diploid strain 007 / 145 was made. This diploid was forced to haploidize on CM + glu + para-fluorophenylalanine (FPA). Although the crinkled morphology is suppressed on FPA medium, a green, pro and paba prototroph, sorbose sensitive and pyridoxin (pyro, from strain 145) auxotroph strain with a crinkled morphology was found. The conidial size was compared to standard duplication and diploid strains using the Coulter Counter, and the conidial size was within the range found for duplication strains. Then this strain was "selfed". Among the descendents no yellow colonies were found (see table 22).

These investigations show that the duplication must be present in strain 007 since the yA, biA, adE and sorA markers are found, although these markers are not phenotypically expressed in strain 007 itself. The yellow marker is located on the normal fragment since upon exchange of chromosome I of strain 007 (in the diploid with 007) this marker is lost. After MMS treatment, predominantly yellow, sorbose resistant, ade and bio prototroph sectors are found, thus yellow must be in coupling with sorA, adE^+, and biA^+. The genotype of strain 007 is concluded to be:



5.4 Localization of the sorA gene

Since the exact position of the sorA gene was not known, the cross 005 x 141 was made. The offspring was grown on supplemented medium (SM) which consisted of MM + paba, bio, ribo, thi, pyro, ade, meth and a carbon source. Bio prototrophs were scored on SM + ace in which the biotin was omitted. Sorbose resistance was scored on SM + ace + SOR and pdh mutants on SM + glu. The latter plates were incubated at 42 °C. The results are shown in table 20.

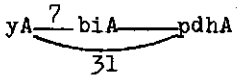
Table 20 Analysis of the cross 005 x 141. Explanation see text.

genotypes among the progeny				recombination		
biA	sorA	pdhA	no.		no.	%
+	+	+	32	biA - sorA	81	26
+	+	-	11	sorA - pdhA	44	14
+	-	+	16	biA - pdhA	99	32
+	-	-	95			
-	+	+	100			
-	+	-	15			
-	-	+	2			
-	-	-	36			
			307			

Legend: see table 19

This cross reveals that the sorA gene is located between the biA and pdhA genes.

Note: Our data of the recombination fraction biA - pdhA of 32 units are inconsistent with the data of Clutterbuck (1981) who gives the following recombination fractions (approx.):



5.5 Handling of strain 007

In order to find the best selective medium for strain 007, some weak carbon sources (lactose, glycerol, arabinose and acetate) in combination with several concentrations of sorbose were compared. Generally, acetate + 18 mM sorbose gave the best differentiation between sorbose resistant and sensitive colonies (data not shown). Only in one case (after azide treatment, incubation method 2) acetate could not be used since the growth of the surviving conidia was inhibited on acetate medium and arabinose was used instead of acetate.

Probably, azide inhibits the enzymes or the induction of enzymes needed for acetate metabolism.

Although the growth of both resistant and sensitive colonies is affected due to the addition of 18 mM sorbose, the difference between the two is evident. For the sensitive colonies are much smaller than the resistant colonies and their conidial colour is much fainter. And when the incubation at 37 °C is alternated with a period at 25 °C, the distinction is even clearer.

The optimal procedure for the incubation of the selection plates containing sorbose was found to be: first 1 day at 37 °C, then 3 days at 25 °C and subsequently another day at 37 °C. All visible yellow colonies are to be counted.

Although the sorbose gene can be readily used for selection procedures, this gene is not very suitable as a marker for characterizing recombinants of strain 007. The two situations are different. Whereas with selection there is a competition between single recombinant conidia and the bulk of test strain conidia, in the case of characterization many identical conidia are always inoculated on the same spot. Complications arise since the difference between resistant and sensitive colonies is relatively small, and there is also the difference between crinkled and wildtype morphology and its intermediate forms, which affects the growth rate.

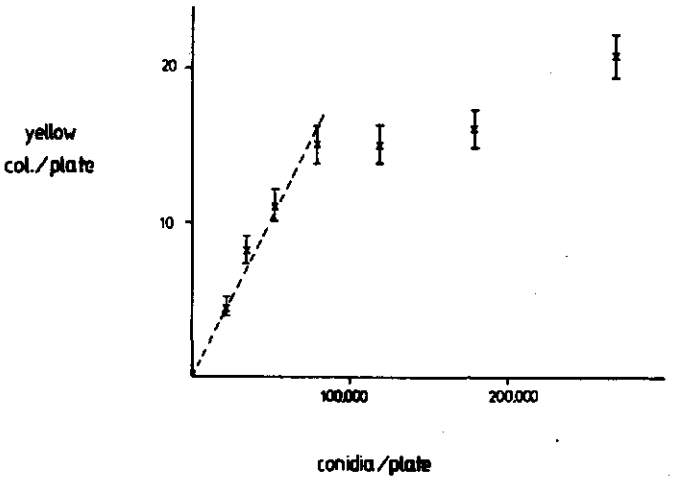
Experiments showed that in the range of 20,000 to 100,000 colonies per plate, the measured mutant frequency is unaffected by the conidial concentrations (fig 4).

5.6 Analysis of the yellow sectors

Isolation and analysis of some of the yellow colonies showed that there are differences between the isolates. Although most of them have wildtype morphology some are still crinkled, indicating that the duplicated fragment has not completely been lost. Nga and Roper (1968) stated that in such cases a partial, likely an interstitial, deletion had occurred. In our study it has been investigated whether mitotic crossing-over can explain this phenomenon.

To this end, 10 yellow colonies (numbered 1 - 10) were isolated from the control plates of a mutagen experiment. Four of them had a crinkled morphology. In the case of a mitotic crossing-over, such a

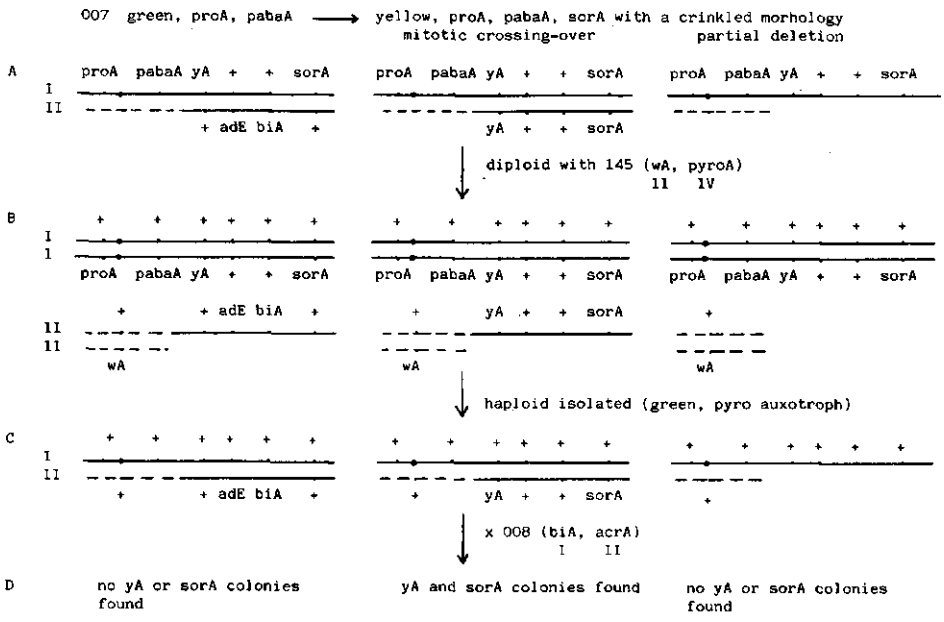
Fig 4 Several dilutions of a conidial suspension of strain 007, treated for 1 hr with 17.7 mM MMS (incubation method), were plated on SM + ace + pro, paba + 18 mM SOR and the numbers of yellow colonies were scored (average of 10 plates).



Legend: x average number of colonies, standard error of mean
- - - - mutation frequencies calculated from the results of the plates with less than 100,000 colonies

yellow, sorbose resistant, crinkled colony would have had two copies of the yA and the sorA allele. In the case of a partial deletion, only one copy (on chromosome I) would have been present (see fig 5 - A). First, starting from single cell colonies, a diploid was made of each isolate with strain 145 (fig 5 - B). Strain 007 itself was also combined with strain 145 into a diploid. These diploids were checked and found to be green, pro, paba and pyro prototroph and sorbose sensitive, and their size was identical to the size of standard diploid strains (measured with the Coulter Counter). These diploids were forced to haploidize by growing them on FPA medium, and in all cases a green, pyro auxotroph haploid strain could be isolated (the pyroA marker is localized on chromosome IV (not shown in fig 5) and was used to distinguish the haploids, since the diploids were pyro prototroph). Comparison of the conidial sizes proved that all were haploids. They must have carried chromosome I of strain 145 because they were pro and paba prototroph, and chromosome II of the isolates since they were not white (fig 5 - C). Then single cell colonies were made. These haploids were crossed to strain 008 and the progeny was checked for the presence of yellow and / or sorbose resistant colonies (fig 5 - D).

Fig 5 Analysis of strain 007 and its yellow, sorbose resistant derivatives (only chromosome I and II are shown). Explanation see text.



When a duplication strain is crossed with a normal diploid strain, it is possible that only part of the ascospores in a cleistothecium is of hybrid origin and that the other ascospores descend from the normal parent only (van de Vate & Jansen, 1978). Therefore, in order to measure the extent to which a cleistothecium was of selfed origin, the number of colonies growing on MM + glu was counted, for a biA$^+$ allele cannot descend from strain 008. About 40% (crinkled isolates) or 25% (normal isolates) of the ascospores of hybrid origin are expected to be both pyro and bio prototroph. The number of viable ascospores was measured by a plating on CM + glu. Sorbose resistant colonies were scored on MM + ace + pyro, bio, ade + SOR. In all cases several dilutions were plated and the numbers of colonies found are given as number / ascospores plated (table 21).

The results, indeed, show that there was much variation in the percentages of pyro and bio prototroph colonies found, as measured by comparing the growth on CM + glu and MM + glu.

Isolates 1, 2, 5 and 6 had a wildtype morphology and did not give recombinants which were yellow or sorbose resistant. Two of them (1 and

Table 21 Analysis of the progeny of the cross 008 x the haploids descending from the diploids of strain 145 / the yellow recombinants of strain 007 or 007 itself. Explanation see text.

isolate no.	morphology of original isolate	growth on MM + glu (c/a)	%	yellow colonies (c/a)	sorbose resistant colonies
007 itself	cr	38/1120	2.2	0/3000	0/11000
1	+	5/33	18	0/4000	0/33
2	+	5/28	18	0/3700	0/300
4	cr	36/1270	2.8	11/3300	6/1270
5	+	59/1320	4.5	0/1370	0/8700
6	+	26/1120	2.3	0/2000	0/7300
8	cr	140/400	35	66/720	99/400
9	cr	43/1790	2.4	6/3210	n.t.
10	cr	n.t.		143/3270	13/189

Legend: c/a colonies per viable ascospores
cr crinkled morphology
\+ wildtype morphology
n.t. not tested

2) reacted like normal wildtypes, but the progeny of the isolates 5 and 6 gave cleistothecia in which only part of the ascospores were of hybrid origin. Since this is the only aspect in which these isolates react like duplication strains, it is likely that only a small part of the duplication is still present. This part does not cover the yA region and is not large enough to create a crinkled morphology, but it is able to disturb, in some way, normal meiotic processes.

Isolates 4, 8, 9 and 10 had a crinkled morphology and all yielded yellow and / or sorbose resistant colonies, indicating that in the original isolates the genetic information located on chromosome I must have been transported to the duplicated fragment by a (normal) crossing-over event.

Isolates 3 and 7 were lost during the genetic manipulations. Strain 007 itself behaved as expected.

Since only few yellow colonies were found in the analysis of the isolates 4 and 9, these two were further investigated as an extra check

Table 22 Percentage of yellow colonies found after plating of ascospores from selfed cleistothecia of haploids isolated out of the diploids: 145 / yellow isolates from strain 007 and 145 / 007 itself. Two cleistothecia were tested from each selfing.

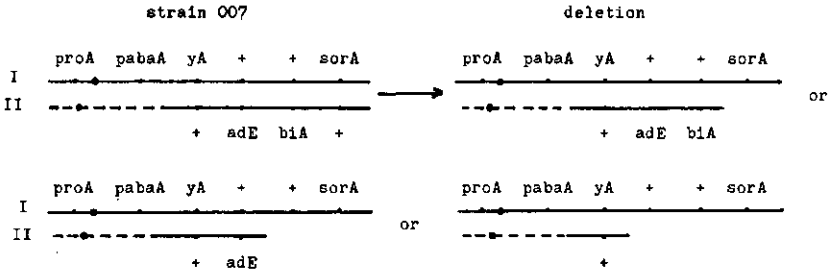
isolate	cleisto-thecium	yellow colonies c/a	%
007	I	0/113	0
	II	0/342	0
4	I	43/440	10
	II	120/1360	9
9	I	62/522	12
	II	19/180	11

Legend: c/a colonies per viable ascospores

by selfing the constructed haploids which had been isolated from the diploids with strain 145. In both cases yellow colonies were observed among the progeny (table 22). This confirms that the isolates 4 and 9 had a duplication fragment, carrying the yA and sorA marker. They must be the result from a crossing-over process in the original duplication strain 007.

5.7 Analysis of the green sorbose resistant sectors

Mutagen treatment yielded, apart from the yellow colonies, also green sorbose resistant colonies. Upon isolation of 20 such colonies, we observed that all had a crinkled morphology, although some had a growth rate which was intermediate between wildtype and duplication strains. The causing mechanism can be a mitotic crossing-over between the yellow and the sorbose marker, or a partial deletion of the translocated fragment. (In the latter case the occurrence of the intermediate growth rate can be easily explained.)



Three green isolates were crossed to strain 145, and the progeny was analysed for the presence of the adE, biA, yA and sorA markers in order to localize the deletion break-point or cross-over point. Yellow colonies were scored on plates CM + glu. For scoring the biA and the adE marker, growth on SM (MM + pro, paba, bio, ade, pyro) + glu was compared to growth on SM + glu in which bio or ade had been omitted. Sorbose resistant colonies were scored on SM + ace + SOR.

As shown in table 23, the cross with isolate 1 yielded colonies with a morphology which was deteriorated compared to the duplication parent. The cause is not known. Isolates 1 and 2 still bear the adE and biA markers, along with the yA marker. The deletion break-point or cross-over is located between the biA and sorA loci. In isolate 3,

Table 23 Crosses of 3 green sorbose resistant sectors of strain 007 x strain 145. Explanation see text.

isolate 1 x 145 phenotypes					colony morphology wild-type	duplication	aberrant*	total
w	y	ad	bi	sor				
w	?	+	+	+	7	19	15	41
w	?	+	+	-	6	6	2	14
w	?	+	-	+	3	0	2	5
w	?	+	-	-	0	0	1	1
w	?	-	-	+	1	0	0	1
+	+	+	+	+	6	5	6	17
+	+	+	+	-	3	8	0	11
+	+	+	-	+	1	0	0	1
+	+	+	-	-	0	0	1	1
+	+	-	-	+	1	0	0	1
+	y	+	+	-	2	4	3	9
+	y	+	-	+	0	0	1	1
+	y	+	-	-	2	0	0	2
					32	42	31	105

* In this cross colonies with an aberrant morphology have been observed: they were much smaller than colonies of the duplication parent.

isolate 2 x 145 phenotypes					colony morphology wild-type	duplication	total
w	y	ad	bi	sor			
w	?	+	+	+	21	14	35
w	?	+	+	-	4	1	5
w	?	+	-	+	2	2	4
w	?	-	+	+	1	0	1
+	+	+	+	+	11	21	32
+	+	+	+	-	0	1	1
+	+	-	-	+	5	0	5
+	y	+	+	+	6	0	6
+	y	+	+	-	10	0	10
					60	39	99

isolate 3 x 145 phenotypes					colony morphology wild-type	duplication	total
w	y	ad	bi	sor			
w	?	+	+	+	23	15	38
w	?	+	+	-	10	8	18
w	?	-	+	-	1	0	1
+	+	+	+	+	14	8	22
+	+	+	+	-	6	8	14
+	y	+	+	+	5	0	5
+	y	+	+	-	6	0	6
					65	39	104

Legend: + wildtype growth or colour
- mutant growth
y yellow colour
w white colour

the adE marker is still present, but the biA marker is not recovered. Only low numbers were analysed, but since the adE marker is nearer to the translocation break-point than the biA marker, more biotin than adenine auxotroph colonies are expected (and indeed found in the cross with the isolates 1 and 2). Thus, it is likely that in the case of isolate 3 the deletion break-point or cross-over is located between adE and biA.

Conclusions whether a crossing-over or a deletion has occurred would only be speculative.

5.8 Results

Several chemicals have been tested with strain 007, with both incubation method 2 and 4. The results are shown in the tables 24 - 38 and the information is given in essentially the same way as for the point mutations (see 4.2). Method 4 generally yielded, compared to method 2, much higher numbers of yellow colonies in the controls. Apparently there is a selective advantage for the yellow colonies when grown on CM + glu.

Methylmethanesulfonate and butadienediepoxide gave comparable results and were both very potent inducers of yellow colonies, both when method 2 and 4 were used (tables 24, 25, 31 and 32). The selectivity factor s was always smaller than 1 and the mutagenicity factor m greater than 1.

Nitrite, when tested with method 2 at pH 4.5, was found to induce yellow colonies only at the highest concentrations (table 26). With method 4, the frequency of the yellow colonies was not enhanced, but calculation of the m and s factors indicated that s was always lower than 1 and was extremely low at the highest concentration NO2 tested. Correspondingly, m was often higher than 1 and became greater by

Table 24 Number of yellow colonies per 10^4 survivors after treatment of conidia of strain 007 with methylmethanesulfonate for 1 hr at 37 °C in liquid suspension, pH 6.8 (incubation method 2).

experiment	conc. (mM)	surv. %	no.	freq.	s.d.	regression y = ax
1 nrem	0	100	45	0.41	0.08	
	5.9	72	43	0.47	0.10	
	11.7	100	86	1.0 *	0.2	a=-4.6
	17.6	83	37	1.9 *	0.4	r=-0.86
	23.5	58	188	3.6 *	0.7	
2 nrem	0	100	25	0.20	0.04	
	17.2	77	22	1.1 *	0.2	a=-2.8
	22.9	49	52	2.3 *	0.5	r=-0.996
	27.6	48	58	2.1 *	0.4	
3 nrem	0	100	18	0.52	0.12	
	23.2	79	40	1.2 *	0.2	a=-3.2
4 rem	0	100	11	0.67	0.20	
	23.5	57	37	1.6 *	0.3	a=-2.2

Legend to the tables 24-30:

- rem germination inhibitor removed
- nrem germination inhibitor not removed
- s.d. standard deviation
- * significantly different from the control value at P<0.05, one-sided
- a slope of the regression y = ax, in which y represents the induced mutation frequency diminished with the control value, and x is ln(survival rate)
- r correlation coefficient

Table 25 Number of yellow colonies per 10^4 survivors after treatment of conidia of strain 007 with butadienediepoxide for 1 hr at 37 °C in liquid suspension, pH 6.8 (incubation method 2).

experiment	conc. (mM)	surv. %	no.	freq.	s.d.	regression y = ax
1 nrem	0	100	25	0.20	0.04	
	3.2	100	99	1.0 *	0.21	
	6.4	57	152	1.7 *	0.4	a= -5.0
	9.7	86	237	3.4 *	0.7	r= -0.91
	12.9	37	282	6.8 *	1.4	
2 nrem	0	100	24	0.21	0.04	
	12.9	61	473	6.4 *	1.3	a=-12.5
3 nrem	0	100	34	0.30	0.06	
	6.4	41	63	0.87*	0.18	a= -1.9
	9.8	52	96	2.2 *	0.5	r= -0.76
	12.9	66	104	2.5 *	0.5	
4 rem	0	100	11	0.67	0.20	
	15.5	67	72	2.6 *	0.5	a= -4.7
5 rem	0	100	13	0.27	0.07	
	26.9	64	76	1.8 *	0.4	
	26.9	67	60	3.7 *	0.8	a= -1.4
	33.6	33	211	2.8 *	0.6	r= -0.62
	33.6	22	174	3.2 *	0.7	
6 rem	0	100	28	0.26	0.05	
	26	42	49	3.6 *	0.7	a= -2.8
	32.5	37	45	2.5 *	0.5	r= -0.90

Legend: see table 24

Table 26 Number of yellow colonies per 10^4 survivors after treatment of conidia of strain 007 with sodium nitrite for 1 hr at 37 °C in liquid suspension, pH 4.5 (incubation method 2).

experiment	conc. (mM)	surv. %	no.	freq.	s.d.	regression y = ax
1 nrem	0	100	14	0.31	0.08	
	11	85	19	0.75 *	0.16	a=-2.4
	20	71	14	0.27	0.07	r=-0.95
	28	35	38	3.1 *	0.6	
2 nrem	0	100	39	0.63	0.13	
	22	80	55	0.63	0.13	a=-0.82
	28	44	72	1.2	0.25	r=-0.996
	33	8	32	2.7 *	0.6	
3 nrem	0	92	19	0.27	0.06	
	0	108	21	0.2	0.05	
	20	97	35	0.56	0.11	
	29	52	55	0.94 *	0.19	a=-1.4
	29	62	30	0.56	0.12	r=-0.98
	39	32	109	2.0 *	0.4	
	39	29	62	2.0 *	0.4	

Legend: see table 24

Table 27 Number of yellow colonies per 10^4 survivors after treatment of conidia of strain 007 with sodium azide for 1 hr at 37 °C in liquid suspension, pH 4.5 (incubation method 2).

experiment	conc. (μM)	c surv. %	d no.	freq.	s.d.	regression y = ax
1 rem	0	100	14	0.78	0.21	
	62	73	11	0.6	0.2	a=0.04
	77	20	25	0.70	0.14	r=0.41
2 rem	0	100	74	0.45	0.09	
	60	32	58	0.92	0.19	a=-1.0
	88	9.5	50	3.2 *	0.7	r=-0.96
3 rem	0	100	7	0.4	0.1	a=-1.5
	104	0.7	61	9.1 *	1.9	r=-0.985
	120	0.4	89	7.2 *	1.7	
4 rem	0	100	267	1.3	0.3	
	81	11	101	0.80	0.16	
	96	4.5	63	1.2	0.3	a=-0.135
	96	2.3	45	2.1	0.5	r=-0.79
	111	4.4	103	2.0	0.4	

Legend: see table 24, except for:
c survival was calculated after filtration and washing of the conidia since the residual N3 inhibited the growth of the conidia in the dilutions of the test suspension
d mutants were scored on arabinose medium since the growth was inhibited on acetate in spite of the washing and filtration step

Table 28 Number of yellow colonies per 10^4 survivors after treatment of conidia of strain 007 with acriflavine for 2 hr at 37 °C in liquid suspension, pH 6.8 (incubation method 2).

experiment	conc. (mM)	surv. %	no.	freq.	s.d.	regression y = ax
1 nrem	0	100	14	0.32	0.09	
	0.1	99	15	0.43	0.11	a=-0.02
	0.2	58	13	0.27	0.08	r=-0.26
	0.4	16	14	0.37	0.10	
2 nrem	0	100	24	0.59	0.12	
	0.29	20	57	1.0	0.21	
	0.29	17	46	0.89	0.18	a=-0.25
	0.44	12	40	1.3 *	0.27	r=-0.97
	0.44	14	48	1.0	0.21	
3 rem	0	100	30	1.0	0.21	
	0.81	1.5	16	3.6 *	0.9	a=-0.62
4 rem	0	100	13	0.39	0.11	
	0.15	14	12	0.63	0.18	a=-0.35
	0.30	3.5	10	1.8 *	0.6	r=-0.97
	0.46	2.1	26	1.8 *	0.4	

Legend: see table 24

Table 29 Number of yellow colonies per 10^4 survivors after treatment of conidia of strain 007 with 9-aminoacridine for 1 or 2 hr at 37 °C in liquid suspension, pH 6.8 (incubation method 2).

experiment	conc. (mM)	surv. %	no.	freq.	s.d.
1 nrem	0	100	16	1.0	0.3
in 2% DMSO	0.4	100	95	0.65	0.13
1 hr	2	62	29	0.92	0.19
2 nrem	0	100	14	0.38	0.10
in 2% DMSO	0.09	58	13	0.46	0.13
2 hr	0.9	30	12	0.90	0.27

Legend: see table 24

Table 30 Number of yellow colonies per 10^4 survivors after treatment of pre-germinated conidia of strain 007 with 9-aminoacridine for 2 hr in liquid MM, pH 6.8, at 37 °C (incubation method 3).

experiment	conc. (mM)	surv. %	no.	freq.	s.d.
1 rem	0	100	11	0.3	0.1
in 2% DMSO	1.4	72	18	0.45	0.11
2 rem	0	100	6	0.2	
in H_2O	0.18	52	6	0.2	
	0.55	100	10	0.4	0.1

Legend: see table 24

increasing NO2 concentrations (table 34).

Azide, tested at pH 4.5 with method 2, increased the number of yellow colonies only significantly at the highest concentration N3 and at a very low survival (table 27). With method 4 no significant change in the frequency of the yellow colonies was seen, although m was often greater than 1 (table 35).

After treatment for two hours in liquid suspension (method 2) with acriflavine, the frequency of the yellow colonies had only been enhanced at the highest concentration ACR and at a very low survival rate (table 28). Using method 4, an extreme selective advantage of the yellow colonies over the parent strain has been seen and the model used to measure m and s was not valid (table 37), since the mutant frequencies found after growth of the artificial suspensions on CM + glu + ACR were too high.

9-Aminoacridine gave no increase of the frequency of the yellow colonies when tested, with method 2 or 3 (tables 29 and 30) in a concentration ranging from 0.09 to 1.4 mM. And, just like ACR, in applying method 4 an extreme selective advantage of the yellow colonies has been observed (table 36).

Table 31 Frequency of yellow colonies (per 10^4) after growth of strain 007 on CM + glu + methylmethanesulfonate (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with yellow recombinants were tested. Incubation for 3 days at 37 °C.

exp.	conc. (mM)	coni-diation	007 no.	007 freq.		s.d.	007 + enrichment no.	freq.	s.d.	m or s	s.d.
1	0	+	138	1.0		0.2					
			62	0.52		0.11					
			80	0.82		0.17					
	0.29	+	501	5.8	*	1.2					
			889	7.7	*	1.6					
			717	6.0	*	1.2					
	0.59	+	995	10	*	2					
			1075	9.1	*	1.9					
			768	9.5	*	2.0					
	1.17	+	1498	16	*	3					
			1724	15	*	3					
			1996	16	*	3					
2	0	+	129	2.4		0.5	1023	143	29		
			129	1.5		0.3	513	74	15		
			57	1.2		0.3	730	88	18		
	0.29	+	316	4.8		1.0	399	43	9		
			289	5.4	*	1.1	712	81	17	m= 6.7	2.1
			304	8.0	*	1.6	417	53	11	s= 0.53	0.11
	0.44	+	511	11	*	2	532	65	13		
			301	8.0	*	1.6	1684	47	10	m=10.6	3.5
			360	6.3	*	1.3				s= 0.47	0.12
	0.59	+	533	13	*	3	508	67	14		
			495	11	*	2	597	74	15	m=10.7	3.4
			413	6.8	*	1.4	341	55	11	s= 0.55	0.12
3	0	+	92	3.1		0.6	123	29	6		
			78	3.4		0.7	151	28	6		
			60	2.1		0.4	179	43	9		
			77	2.6		0.5	150	27	6		
	0.29	(+)	181	7.4	*	1.5	535	25	5		
			178	6.9	*	1.4	460	20	4	m= 4.5	1.6
			195	7.4	*	1.5	119	27	6	s= 0.6	0.15
	0.59	(+)	416	15	*	3	108	35	7		
			344	14	*	3	111	24	5	m=10	4.5
			433	16	*	3	148	33	7	s= 0.54	0.19
	1.17	(-)	118	22	*	5	107	28	6		
			151	40	*	8	154	32	7	g	
			318	34	*	7	158	37	8		
4	0	+	153	6.2		1.3	109	67	14		
			156	6.2		1.3	87	48	10		
	0.29	+	227	8.5		1.8	252	23	5	m= 4.6	2.1
			169	6.5		1.3	188	19	4	s= 0.26	0.09
	0.59	+	120	17	*	4	228	19	4	m=25	25
			275	9.2		1.9	179	15	3	s= 0.08	0.07
	1.22	(+)	171	20	*	4	97	49	10	m= 6.2	3.3
							95	44	9	s= 0.52	0.19

Legend to the tables 31-38:

+	dark-green conidia
(+)	light-green or brown conidia
(-)	conidia only faintly coloured
-	no conidial colour seen
m	multiplying effect on the spontaneous mutant frequency caused by recombinogenicity
s	multiplying effect on the spontaneous mutant frequency caused by selective advantage
s.d.	standard deviation
*	significantly larger than the highest recombinant frequency of the controls ($P<0.05$, one-sided)
e	the difference between the repeats is significant ($P<0.05$, two-sided)
d	the model is not valid since there is too much advantage of the yellow sectors (s would be very much larger than 1)
g	m and s are negative since the mixture yielded less mutants than the original suspension

Table 32 Frequency of yellow colonies (per 10^4) after growth of strain 007 on CM + glu + butadienediepoxide (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with yellow recombinants were tested. Incubation for 3 days at 37 °C.

exp.	conc. (mM)	coni-diation	007 no.	007 freq.	007 s.d.	007 + enrichment no.	007 + enrichment freq.	007 + enrichment s.d.	m or s	s.d.
1	0	+	92	3.1	0.6	123	29	6		
			78	3.4	0.7	151	28	6		
			60	2.1	0.4	179	43	9		
			77	2.6	0.5	150	27	6		
	0.13	+	176	11 *	2	126	27	6		
			205	9.5*	2.0	97	23	5	m= 6.3	2.4
			181	6.5	1.3	108	20	4	s= 0.5	0.14
	0.26	(+)	265	11 *	2	92	29	6		
			275	12 *	2	101	29	6	m= 8.5	3.6
			249	8.9*	1.8	111	13	3	s= 0.45	0.14
	0.52	(-)	33	9.5*	2.0	91	29	6		
			439	22 *	5	144	31	7	m= 14.6	7.7
			513	17 *	6	66	23	5	s= 0.4	0.17
2	0	+	53	1.7	0.4	473	164	34		
			104	2.3	0.5	493	212	44		
	0.13	+	235	8.3*	1.7	240	103	21	m= 7.6	2.6
			199	5.8*	1.2	179	83	17	s= 0.46	0.11
	0.26	(+)	266	10 *	2	130	49	10	m= 31	12
			346	12 *	3	718	38	8	s= 0.17	0.05
	0.52	(-)	352	22 *	5	600	53 e	11	m=101	54
			485	17 *	4	718	21 e	4	s= 0.1	0.04
	0.64	-	336	47 *	10	143	44	9	m=840	1900
			402	25 *	5	182	36	7	s= 0.02	0.05

Legend: see table 31

Table 33 Frequency of yellow colonies (per 10^4) after growth of strain 007 on CM + glu + vinclozolin (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with yellow recombinants were tested. Incubation for 3 days at 37 °C.

exp.	conc. (µM)	coni-diation	007 no.	007 freq.	007 s.d.	007 + enrichment no.	007 + enrichment freq.	007 + enrichment s.d.	m or s	s.d.
1	0	(+)	115	4.7	1.0	638	209	43		
in 0.4%			120	4.4	0.9					
DMSO	5.2	(+)	132	17 *	3	664	360	74		
			103	12 *	2	513	300	62	m=1.5	0.5
			146	15 *	3	15	670	170	s=2.1	0.6
	14	(-)	96	9.2*	1.9	307	240	49		
			108	10 *	2	341	178	37	m=2.4	0.9
						564	145	30	s=0.9	0.2
2	0	(+)	125	4.7e	1.0	94	53	11		
in 0.4%			176	7.2e	1.5	273	43	9		
DMSO			77	2.9e	0.6	365	29	6		
	10.5	(-)	178	13 e	3	194	103	21		
			311	9.0e	1.9	210	94	19	m=0.61	0.12
			187	3.7e	0.8	370	142	29	s=2.8	0.6
	14	(-)	239	23 *	5	477	207	43		
			184	28 *	6	304	139	29	m=1.1	0.4
			162	19 *	4	1873	183	38	s=4.2	1.0
	21	(-)	363	27 *	6	1505	207	43		
			216	27 *	6	437	213	44	m=0.9	0.3
			211	19 *	4	385	287	59	s=5.8	1.3
3	0	(+)	44	1.7	0.4	87	48	10		
in 0.4%			71	3.3	0.7	69	27	6		
DMSO			56	2.0	0.4	13	41	11		
	7	(+)	219	9.3*	1.9	485	321	66		
			77	8.0*	1.6	393	243	50	m=0.52	0.16
			217	7.1*	1.5	275	190	39	s=6.7	1.5
	14	(-)	318	5.5e	1.1	125	119	25		
			56	4.1e	0.8	412	154	32	m=1.3	0.4
			112	20 e*	4	211	127	26	s=3.4	0.8

Legend: see table 31

Table 34 Frequency of yellow colonies (per 10^4) after growth of strain 007 on CM + glu + sodium nitrite (incubation method 4). The pH of the agar was adjusted to 4.5. Both normal conidial suspensions and suspensions artificially enriched with yellow recombinants were tested. Incubation for 3 or 4 days at 37 °C.

exp.	conc. (mM)	coni-diation	007 no.	007 freq.	007 s.d.	007 + enrichment no.	007 + enrichment freq.	007 + enrichment s.d.	m or s	s.d.
1	0	+	52	2.4	0.5	1235	259	53		
(3 days)			417	1.6	0.3	869	186	38		
	0.92	(-)	25	2.1	0.4	49	11	2	m=12.8	4.5
			38	1.6	0.3	30	25	5	s= 0.07	0.02
2	0	+	92	2.3	0.5	214	5.6	1.2		
(4 days)			157	3.8	0.8	310	6.6	1.4		
			92	2.0	0.4					
	0.92	+	87	2.0	0.4	204	3.4	0.7		
			67	1.5	0.3	174	3.5	0.7	m= 0.83	0.42
			80	1.5	0.3	226	5.7	1.2	s= 0.75	0.3
	1.84	(+)	47	0.85	0.18	65	1.3	0.3		
			46	1.0	0.2	59	1.2	0.3	m= 2.6	1.9
			69	0.87	0.18	73	1.5	0.3	s= 0.13	0.08
	2.76	(-)	92	2.8	0.6	96	3.0	0.6	m=37	350
			9	3	0.6	11	3.0	0.6	s= 0.03	0.28
3	0	+	81	2.7	0.6	54	19	4		
(4 days)			74	2.1	0.4					
			73	2.8	0.6					
	0.46	+	33	0.93	0.19	39	13	2.6		
			39	1.0	0.2	117	9.2	1.9	m= 0.74	0.27
			37	1.0	0.2	109	7.2	1.5	s= 0.52	0.15
	0.92	(-)	40	1.3	0.3	74	4.8	1.0		
			38	1.0	0.2	55	3.2	0.7	m= 2.7	1.8
			32	0.66	0.14	43	2.2	0.5	s= 0.14	0.05

Legend: see table 31

Table 35 Frequency of yellow colonies (per 10^4) after growth of strain 007 on CM + glu + sodium azide (incubation method 4). The pH of the agar was adjusted to 4.5. Both normal conidial suspensions and suspensions artificially enriched with yellow recombinants were tested. Incubation for 3 or 4 days at 37 °C.

exp.	conc. (µM)	coni-diation	007 no.	007 freq.	007 s.d.	007 + enrichment no.	007 + enrichment freq.	007 + enrichment s.d.	m or s	s.d.
1	0	+	21	1.4	0.4	189	156	32		
(3 days)			37	1.5	0.3	378	177	36		
			46	1.9	0.4	419	267	55		
	9.5	+	50	3.0	0.6	1047	131	27		
			38	1.5	0.3	1320	165	34	m=1.7	0.5
			41	1.7	0.4	313	162	33	s=0.8	0.15
	19	(+)	69	2.2	0.5	1057	138	28		
			38	1.5	0.3	1267	127	26	m=1.5	0.4
			32	1.2	0.5	1438	146	30	s=0.7	0.14
2	0	+	74	2.1	0.4	54	19	4		
(4 days)			81	2.7	0.6					
			73	2.8	0.6					
	9.5	+	108	3.3	0.7	46	17	4		
			178	4.6	0.9	63	19	4	m=1.6	0.6
			80	2.6	0.5				s=0.85	0.27
	19	+	71	2.4	0.5	74	14	3		
			58	1.8	0.4	49	18	4	m=1.0	0.37
			49	1.9	0.4				s=0.8	0.25
3	0	+	92	2.3	0.5					
(4 days)			157	3.8	0.8					
			92	2.0	0.4					
	19	+	88	2.0	0.4					
			96	2.1	0.4					

Legend: see table 31

Table 36 Frequency of yellow colonies (per 10^4) after growth of strain 007 on CM + glu + 9-aminoacridine (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with yellow recombinants were tested. Incubation for 3 days at 37 °C.

exp.	conc. (mM)	coni-diation	007 no.	007 freq.	007 s.d.	007 + enrichment no.	007 + enrichment freq.	007 + enrichment s.d.	m or s	s.d.
1	0	+	201	3.9	0.8	524	9.9e	2.0		
in H_2O			126	7.0	1.4	54	28 e	6		
			58	4.2	0.9	321	29 e	6		
	0.52	(-)	373	25 e*	5	324	145 e	30		
			89	73 e*	15	647	234 e	48	m= 1.0	0.4
			142	83 e*	17	16	385 e	79	s=11.5	3.1
	1.04	-	109	38 e*	8	206	730 e	150		
			85	49 e*	10	352	166 e	34	b	
			73	134 e*	28	25	3250 e	700		
2	0	+	120	2.1	0.4	421	96	20		
in H_2O			48	1.8	0.4	323	97	20		
			54	2.5	0.5	459	75	15		
	0.24	(+)	120	8.4*	1.7	563	239	49		
			95	9.7*	2.0	567	390	80	m= 1.2	0.4
			132	9.7*	2.0	484	360	74	s= 3.7	0.8
	0.49	(-)	82	164 e*	34	460	8000	1600		
			17	64 e*	13	455	9100	1900	b	
	0.97	(-)	4	49 e*		60	9000	1900		
			85	1000 e*	200	37	9500	2000	b	
			57	690 e*	140	54	7500	1500		

Legend: see table 31

Table 37 Frequency of yellow colonies (per 10^4) after growth of strain 007 on CM + glu + acriflavine (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with yellow recombinants were tested. Incubation for 3 days at 37 °C.

exp.	conc. (µM)	coni-diation	007 no.	007 freq.	007 s.d.	007 + enrichment no.	007 + enrichment freq.	007 + enrichment s.d.	m or s	s.d.
1	0	+	92	3.1	0.6	123	29	6		
			78	3.4	0.7	151	28	6		
			60	2.1	0.4	179	43	9		
			77	2.6	0.5	150	27	6		
	18	+	66	2.0	0.4	164	22	5		
			38	1.8	0.4	207	36	8	m= 0.83	0.25
			51	2.3	0.5	102	24	5	s= 0.87	0.18
2	0	+	184	4.0	0.8	1240	223	46		
			278	3.9	0.8	1000	128	26		
	23	(-)	1125	518 *	107	118	8700	1800	b	
			890	223 *	46	102	7700	1600		
3	0	+	201	3.9	0.8	524	10e	2		
			126	7.0	1.4	24	28e	6		
			58	4.2	0.9	321	29e	6		
	20	-	94	8.0	1.6	45	385	79		
			60	14	3	29	525	108	m= 0.08	0.03
			227	17 *	4	72	502	165	s=32	8
	24	-	298	6.5e	1.3	140	2300	470		
			426	34 e*	7	154	4400	900	b	
			142	4.6e	1.0	143	2200	450		
	29	-	112	8.4e	1.7	240	6200	1280		
			66	111 e*	23	251	7000	1440	b	
						60	4900	1010		

Legend: see table 31

Table 38 Frequency of yellow colonies (per 10^4) after growth of strain 007 on CM + glu + chloralhydrate (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with yellow recombinants were tested. Incubation for 3 days at 37 °C.

exp.	conc. (mM)	coni-diation	007 no.	007 freq.	007 s.d.	007 + enrichment no.	007 + enrichment freq.	007 + enrichment s.d.	m or s	s.d.
1	0	+	60	1.9 e	0.4	393	70	14		
			76	2.1 e	0.4	263	48	10		
			25	0.86e	0.18	224	37	8		
	1.5	(+)	28	1.0	0.2	192	48	10		
			25	0.74	0.15	206	34	7	m=0.64	0.2
						198	42	9	s=0.81	0.2
2	0	+	87	2.9	0.6	114	54	11		
			83	3.0	0.6	106	51	11		
			49	3.0	0.6	93	43	9		
	3.0	(+)	135	5.4	1.1	106	86	18		
			146	5.7	1.2	94	55	11	m=1.4	0.4
			92	5.5	1.1	103	67	14	s=1.4	0.3
	4.5	(+)	114	3.7	0.8	97	57	12		
			170	5.3	1.1	121	57	12	m=1.7	0.5
			137	5.7	1.2	155	38	8	s=1.0	0.2
	6.0	(+)	50	2.2	0.5	180	20	4		
			40	1.7	0.4	98	13	3	m=2.1	0.7
			56	2.4	0.5	183	20	4	s=0.34	0.08
	7.5	(+)	32	0.69	0.14	40	5.9	1.2		
			28	0.78	0.16	44	4.4	0.9	m=4.2	1.4
			23	1.5	0.3	25	3.7	0.8	s=0.08	0.02

Legend: see table 31

Vinclozolin was only tested using method 4 (table 33). The number of yellow colonies was always enhanced, but the effect was on s. In one experiment, m was found to be more enhanced than s, but this result was not reproduced in the other experiments and therefore believed to be an artefact.

Chloralhydrate, too, was only tested with method 4 (table 38), and the number of yellow colonies was certainly not increased. However, s was found to be lower than 1 and decreased with increasing concentrations of CH. And m was higher than 1 and seemed to increase with increasing concentrations of CH.

5.9 Preliminary conclusions

The presence of the sorA2 gene in strain 007 reduces the number of plates needed to measure the instability of a duplication strain.

Mitotic crossing-over can lead to yellow colonies which can only be distinguished from deletion products if special care is taken to observe the morphology (crinkled or normal) of the recombinants.

MMS and BED are very potent inducers of yellow colonies.

NO2 (only tested at pH 4.5) is a weak inducer of yellow segregants when tested with method 2 and method 4.

N3 (only tested at pH 4.5) induces segregants only at a very low survival rate using method 2. And with method 4 no significant activity is found.

ACR is a weak inducer of yellow segregants when tested with method 2, and 9AA was not mutagenic with either method 2 or 3. Both agents cannot be tested with method 4 because of the extreme selective advantage of the yellow colonies.

VIN has only been tested with method 4 and the main effect is a selective advantage of the segregants.

CH shows some mutagenic activity when tested with method 4.

The trends of mutagen response are almost identical to those of point mutations (see general discussion),

6 MITOTIC CROSSING-OVER AND NON-DISJUNCTION

6.1 General remarks

In diploid strains of Aspergillus several processes during mitosis can lead to sectors with non-parental genotype. Chemicals of different classes enhance this sectoring. If suitable markers are present the process involved can be investigated.

A mitotic crossing-over (fig 1A), the process most frequently found, leads to homozygosity of the markers distal to the cross-over by which the properties of recessive genes come to expression. However, a deletion (fig 1B) can lead to phenotypically identical colonies. In this study no attempt has been made to differentiate between these two processes.

A non-disjunction (fig 1D) can lead to diploids with two identical homologues of one chromosome. In this case all recessive genes on both sides of the centromere will be expressed. Also haploids are formed in which recessive genes of all chromosomes will be expressed.

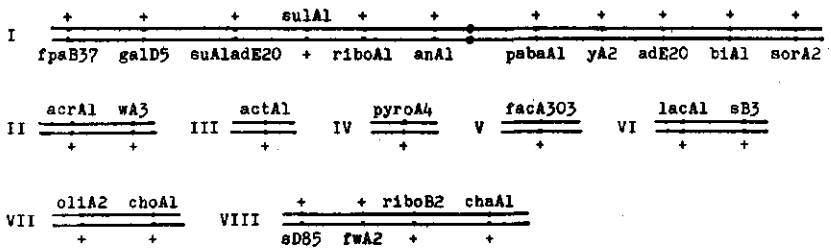
The gene conversion frequency (fig 1C) is very low in Aspergillus (de Bertoldi et al., 1980). Thus, mutant sectors will not likely be caused by this process.

In our study, several methods for measuring the frequency of crossing-over and non-disjunction during mitosis have been compared.

6.2 Mitotic segregation

When a diploid strain heterozygous for a conidial colour marker is grown on complete medium, spontaneously sectors with non-parental conidial colour arise (mitotic segregation). Chemical compounds like benomyl and actinomycin D are known to induce an increased sectoring (Kappas, 1978). The mechanism involved can only be revealed after isolation and genetic analysis of the sectors.

In the present study strain D4 has been used carrying 4 colour markers: y (yellow), w (white), cha (chartreuse) and fw (fawn). The genotype of strain D4 is:



The following test procedure (incubation method 1) was used: Firstly, conidia were plated in low density on CM + glu, and after 24 hr incubation at 37 °C, pieces of agar and mycelium were transferred to fresh plates of CM + glu + the chemical to be tested. Only 5 colonies were grown on each plate. After 7 days incubation at 37 °C, all visible sectors with a mutant conidial colour have been counted.

Using this system, vinclozolin and carbendazim were found to enhance the sectoring of strain D4 (table 39). Here no attempt was undertaken to analyse the sectors. Thus the inducing mechanism is not known.

Table 39 Number of sectors with a recessive conidial colour found after growth of 50 colonies of strain D4 (5 col/plate) for 7 days on CM + glu + carbendazim or vinclozolin. As a solvent ethanol was used.

	sectors in 50 colonies	
control (ethanol)	26	
control (ethanol)	21	
0.52 µM carbendazim	42	
1.31 µM carbendazim	60	*
2.10 µM carbendazim	127	*
0.98 µM vinclozolin	30	
1.96 µM vinclozolin	44	*
3.90 µM vinclozolin	82	*

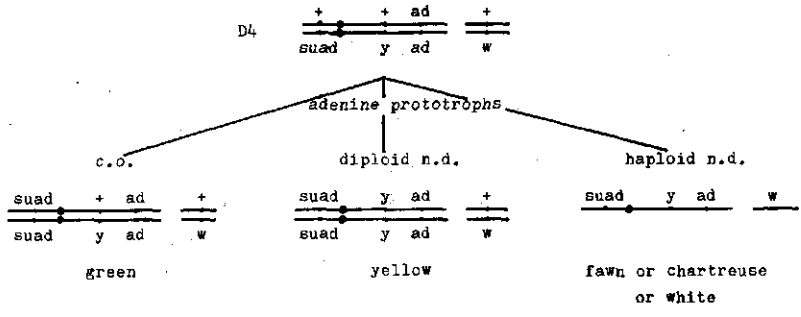
Legend: * significantly enhanced compared to the controls (t-test, $P<0.05$, one-sided)

When it is intended to discriminate between the inducing mechanisms, it is more convenient to select a specific recombinant than to isolate recombinants at all possible loci. Strain D4 offers several possibilities.

6.3 Selection of adenine prototrophs

The suppressor adenine system in strain D4 can be used to select recombinants. Strain D4 is homozygous for the adE20 allele and heterozygous for its suppressor allele suAladE20. Since this suad allele is

recessive, strain D4 grows only poorly on medium without adenine. Diploid segregants homozygous for the suad allele or haploids carrying the suad allele grow normally in absence of adenine, although the conidial colour is somewhat faint. The suppressor controlled adenine prototrophs produced by a crossing-over are green. Diploid non-disjunction products, however, are yellow and haploids have a fawn or chartreuse, or a white colour. Schematically:



As selective medium, MM + glu + growth factors and amino acids was used (further on called SM + glu - ade). The growth factors and amino acids used were: paba, bio, ribo and thi (needed for the diploid and haploid recombinants), and pyro, meth and cho (for the haploids only). Three plating procedures were compared: 1 the conidial suspension was plated on SM + glu - ade. 2 the conidial suspension was mixed with 5 ml SM + glu - ade (melted and cooled down to 50 °C) and poured out on a thin plate SM + glu - ade. 3 the conidial suspension was mixed with 5 ml SM + glu - ade (melted and cooled down to 50 °C), poured into petri-dishes, and when these plates had become solid, a second layer of ca. 15 ml SM + glu - ade was laid on the first. The incubation was for 3 - 4 days at 37 °C.

When procedure 1 was used, segregants and parental colonies could be distinguished, but procedure 2 was found to give clearer results since the distribution of the colonies over the plates was better. Procedure 3 could not be used, since too many colonies started to grow through the agar as a result of the leaky growth.

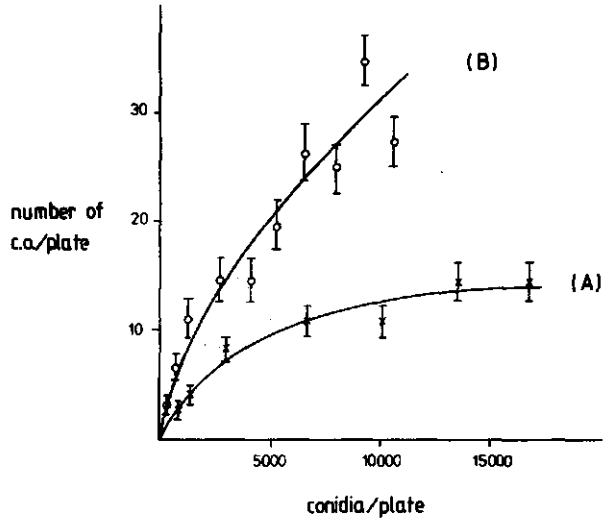
However, even when plating-procedure 2 was used, the scoring of the recombinants was not as easy as expected. When the conidia were first treated in liquid suspension (incubation method 2 or 3) with a mutagen (MMS), the morphology of the adenine prototroph colonies on SM + glu -

ade was different from the morphology of the adenine prototroph colonies found after growth of untreated conidia on the same medium.
-A- Untreated conidia gave two distinct types of green, adenine prototroph colonies in a background of aconidiate mycelium of strain D4: large, round colonies and smaller, irregular shaped colonies. Intermediate forms were rare. Probably those conidia in which the crossing -over took place before or shortly after the inoculation on the selection plates gave large colonies. During the leaky growth of unchanged D4 mycelium, however, new crossing-overs occurred at a later moment and therefore gave rise to the small, irregular shaped colonies. The colonies with recessive conidial colour were usually somewhat smaller than the green colonies. It would be reasonable to count only the large green colonies and the (somewhat smaller) largest colonies with other conidial colour.
-B- After mutagen treatment both types of adenine prototroph colonies were found more often than in the control, and the difference between the large and the small, irregular shaped colonies was reduced. Some intermediate forms (large, with a reduced size) were seen. Here it was difficult to discriminate between the large and the smaller colonies, and a somewhat arbitrary minimal colony size had to be the limit. And since the colonies arising after non-disjunctions were often smaller than the green colonies, different criteria were needed for the green as for the yellow, chartreuse, fawn and white colonies.

After treatment of conidia of strain D4 in liquid suspension (incubation method 2), it was observed that the frequency of the green adenine prototrophs depended on the number of conidia inoculated per plate (fig 6). As expected, when too many conidia (more than ca. 15,000) were inoculated per plate, the crossing-over frequency was underestimated, since neighbouring colonies were counted as one. However, at a very low conidial concentration, the crossing-over frequency was overestimated. The colonies of D4 had probably more space and nutrients for leaky growth, and thus they had more opportunity for recombination. And these resulting colonies were so large that they were included in the count, in spite of the attempted differentiation between larger and smaller, irregular shaped colonies.

Thus recombinant frequencies can only be compared if they result from selection plates inoculated with a conidial concentration within a certain range (7,000 - 12,000 conidia / plate).

Fig 6 Average number of adenine prototroph crossing-over products found in dependence of the number of conidia of strain D4 inoculated per plate SM + glu - ade. Both untreated (A) and MMS-treated (B) conidia were plated (incubation method 2).



Legend: O average number of crossing-overs, after treatment with MMS
X average number of crossing-overs, untreated conidia

Several experiments (incubation method 2) were performed in which adenine prototrophs were scored. The accumulated results of the controls gave a spontaneous frequency of 3.5 crossing-over products per 10^4 conidia. Thus the real crossing-over frequency is 0.07%, which is lower than 0.1 to 0.3% as estimated by Käfer (1977). We also found 0.25 diploid and 0.43 haploid non-disjunction products per 10^4 conidia.

When incubation method 2 or 3 is used, the suppressor adenine system is not very suitable for measuring the non-disjunction frequency, firstly because this frequency is very low compared to the frequency of the crossing-overs and secondly due to the small colony size. When method 4 is used, the colonies are larger and the plates are easier to score since the difference between the larger and the smaller, irregular shaped adenine prototroph colonies is clearer.

6.4 Tests with the suppressor adenine system

Three different chemicals were tested with this system (incubation method 4). The results are given in the tables 40 - 42. Both the green

Table 40 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D4 on CM + glu + methylmethanesulfonate (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with recombinants were tested. Incubation for 3 days at 37 °C.

exp.	conc. (mM)	coni-diation	crossing-over (green) D4 no.	freq.		s.d.	non-disjunction (yellow) D4 no.	freq.		s.d.	D4 + enrichment no.	freq.		s.d.	m or s	s.d.
1	0	+	54	6.8		1.4	13	1.6		0.4	59	15		3		
			27	4.8		1.0	11	2.0		0.6	87	17		4		
			34	4.3		0.9	5	0.6			82	13		3		
	0.29	+	100	53	*	11	9	4.8		1.6	41	8.5		1.8		
			113	78	*	16	4	2.7			16	11		3	m= 3.7	2.0
			125	43	*	9	5	1.7			24	15		3	s= 0.6	0.2
	0.59	+	218	115	*	24	13	6.9	*	1.9	14	9.7		2.6		
			204	79	*	16	18	7.0	*	1.6	14	11		3	m=15	11
			159	71	*	15	10	4.4		1.4	12	9.4		2.7	s= 0.3	0.2
	1.17	+	314	108	*	22	29	9.9	*	2.0	25	14		3		
			348	126	*	26	24	8.7	*	1.8	24	18		4	m=21	15
			291	127	*	26	30	13	*	3	19	14		3	s= 0.4	0.2
2	0	+	18	4.4		1.0	8	2.0		0.7	9	4.7		1.6		
			15	4.6		1.2	8	2.5		0.9	7	2.3				
							10	4.4		1.4	9	4.0		1.3		
	0.12	+	72	25	*	5	7	2.4			11	8.4		2.5		
			70	26	*	5	9	3.3		1.1	5	2.8			m= 0.29	0.53
			77	24	*	5	5	1.5			3	1.9			s= 2.8	4.6
3	0	n.s.	9	2.0		0.7	8	1.8		0.6						
			13	1.4		0.4	9	1.0		0.3						
	0.59	n.s.	459	97	*	20	29	6.4	*	1.3						
			550	88	*	18	43	6.9	*	1.4						

Legend to the tables 40-42 and 44-51:

+	dark-green conidia
(+)	light-green conidia
(-)	conidia only faintly coloured
-	no conidial colour seen
m	multiplying effect on the spontaneous recombination frequency caused by recombinogenicity
s	multiplying effect on the spontaneous recombination frequency caused by selective advantage
s.d.	standard deviation
n.s.	not scored
*	significantly larger than the highest mutant frequency of the controls ($P<0.05$, one-sided)
e	the difference between the repetitions is significant ($P<0.05$, two-sided)
g	m and s are negative since the mixture yielded less mutants than the original suspension

(crossing-overs) and other (non-disjunctions) recombinants on each plate were counted. The information is given in essentially the same way as for the point mutations (see chapter 4.2).

After growth on MMS containing medium, the frequency of the crossing-over products was greatly increased (table 40). The frequency of colonies with other conidial colour was also enhanced. And m was much higher than 1, but it seems possible that, in view of the high number of crossing-overs, part of the yellow, white, chartreuse and fawn colonies resulted from a double crossing-over.

After growth on vinclozolin containing medium, the frequency of the crossing-over products was not enhanced and the number of non-disjunction products was only slightly increased (table 41). Here only normal conidial suspensions were tested, so m and s were not calcu-

Table 41 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D4 on CM + glu + vinclozolin (incubation method 4). Only normal conidial suspensions and were tested. Incubation for 3 days at 37 °C.

			crossing-over (green) D4			non-disjunction (yellow) D4		
exp.	conc. (µM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.
1	0	+	101	9.0	1.9	22	2.0	0.4
in 0.1%			67	6.8	1.4	8	0.8	0.3
DMSO			67	3.5	0.7	12	1.0	0.3
	1.7	+	67	7.9	1.6	32	3.8	0.8
			45	5.1	1.1	17	1.9	0.5
			48	5.3	1.1	12	1.3	0.4
	3.5	+	57	7.4	1.5	25	3.2	0.7
			125	13	3	42	4.2 *	0.9
			146	13	3	35	3.2	0.7
	7.0	(+)	91	8.9	1.8	30	3.0	0.6
			97	9.3	1.9	66	6.3 *	1.3
			108	10	2	60	5.6 *	1.2
	14	(-)	24	8.2	1.7	18	6.2e*	1.5
			17	6.7	1.6	28	11 e*	2
			24	8.1	0.6	13	1.7e	0.5
2	0	+	47	6.0	1.2	19	2.4	0.6
in 0.2%			70	6.6	1.4	15	1.4	0.4
DMSO	1.7	+	98	8.5	1.8	26	2.3	0.5
			49	7.3	1.5	15	2.2	0.6
	3.5	+	22	2.7	0.6	19	2.3	0.5
			41	4.4	0.9	31	3.3	0.7
	7.0	(+)	62	8.2	1.7	46	6.1 *	1.3
			12	9.7	2.8	5	4.2	

Legend: see table 40

Table 42 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D4 on CM + glu + chloralhydrate (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with recombinants were tested. Incubation for 3 days at 37 °C.

			crossing-over (green) D7			non-disjunction (yellow) D7			D7 + enrichment				
exp.	conc. (mM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d	no.	freq.	s.d.	m or s	s.d.
1	0	+	54	6.8	1.4	13	1.6	0.4	59	15	3		
			27	4.8	1.0	11	2.0	0.6	87	17	4		
			34	4.3	0.9	5	0.6		82	13	3		
	6.0	(-)	10	9.8	3.1	68	67 *	14	203	147	30		
			21	14	3	169	108 *	22	114	136	28	m=23	18
			60	9.3	1.9	672	104 *	21	118	112	23	s= 2.9	1.8
2	0	+	25	3.3e	0.7	2	0.3		54	12	2		
			19	2.1e	0.5	1	0.1		53	14	3		
			19	4.6e	1.1	6	1.4		66	11	2		
			35	5.8e	1.2	2	0.3		67	14	3		
			28	4.9e	1.0	4	0.7		62	13	3		
			21	4.1e	0.9	4	0.8		42	15	3		
	8.9	(-)	17	14 *e	4	124	105 *	22	145	730e	150		
			15	5.9e	1.5	151	59 *	12	250	690e	140	m= 4.7	1
			13	4.3e	1.2	169	56 *	12	191	124e	26	s= 5.8	1.2
			7	3.2e		137	62 *	13	188	171e	35		
3	0	+	75	7.2	1.5	17	1.6	0.4					
	4.0	(+)	47	4.4	0.9	115	11	2					
			37	4.2	0.9	48	5.4	1.1					
	6.0	(-)	48	5.6e	1.2	131	15	3					
			131	16 *e	3	196	24 *	5					

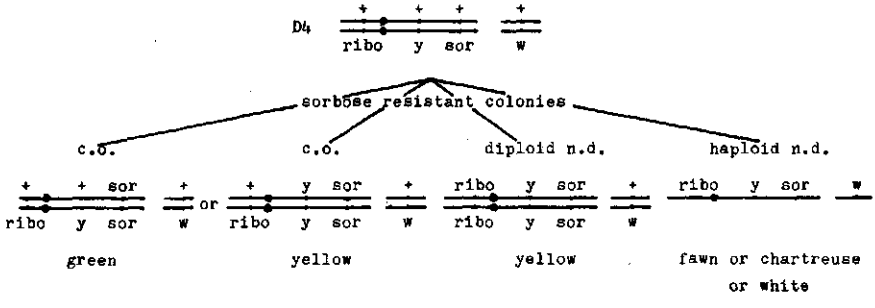
Legend: see table 40

lated.

After growth on chloralhydrate containing medium, no influence on the crossing-over frequency was found, but there were significantly more non-disjunction products (table 42). The m factor was always higher than 1.

6.5 Selection of sorbose resistant recombinants

Another possible way to select recombinants is to use the sorA2 allele for resistance to sorbose in strain D4. Strain D4 itself grows only poorly on media supplemented with sorbose. Non-disjunction and crossing-over products, however, can be homozygous for the sorA allele, and then they show good growth and sporulation. Using this system, crossing-over and diploid non-disjunction products can, unfortunately, not be easily distinguished, since also crossing-overs can give yellow colonies. Only after isolation of the recombinants and analysis of the markers on the other arm of the first chromosome, the mechanism involved can be revealed. Haploid non-disjunction products can be recognized directly because of the chartreuse or fawn, or white colour of their conidia. Schematically:

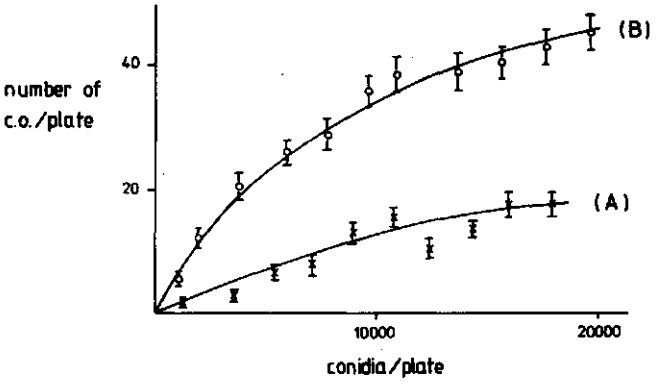


As selective medium MM + ace + SOR, supplemented with growth factors and amino acids: paba, bio, ribo, thi, ade, pyro, meth and cho was used (further on called SM + ace + SOR). Acetate was used since sorbose resistance can only be scored in the presence of a weak carbon source (see chapter 5.5). Again the three plating procedures were compared: 1 the conidial suspension was plated on SM + ace + SOR. 2 the conidial suspension was mixed with 5 ml SM + ace + SOR (melted and cooled down to 50 °C) and poured out on a thin plate SM + ace + SOR. 3 the conidial suspension

was mixed with 5 ml SM + ace + SOR (melted and cooled down to 50 °C), poured into petridishes and when these plates had become solid, a second layer of ca. 15 ml SM + ace + SOR was laid on the first. The incubation was for 3 - 4 days at 37 °C.

Procedure 1 was not successful. There was too much variation in the growth rate of the recombinants, and the difference between the sorbose resistant and the sorbose sensitive colonies was relatively small. Also when procedure 2 was used, too many colonies grew through the agar. Upon isolation and analysis, several of them were found to be sorbose sensitive. Procedure 3 gave the best differentiation between sorbose resistant and sorbose sensitive colonies, since all colonies had to grow through the agar, and this was such a barrier that resistant colonies were seen only. Upon variation of the sorbose concentration and the period of time between the inoculation of the conidia and the pouring of the second layer, it was observed that the best conditions were: Firstly, the conidia were mixed with 5 ml SM + ace (melted and cooled down to 50 °C) and plates were poured. These plates were incubated for 24 hours at 37 °C to allow germination of the conidia. Then a second layer of ca. 12 ml SM + ace + 18 mM SOR was laid on top of the first and again, these plates were incubated for 3 days at 37 °C.

Fig 7 Average number of sorbose resistant crossing-over products found in dependence of the number of conidia of strain D4 inoculated per plate SM + ace + SOR. Both untreated (A) and MMS-treated (B) conidia were plated (incubation method 2).

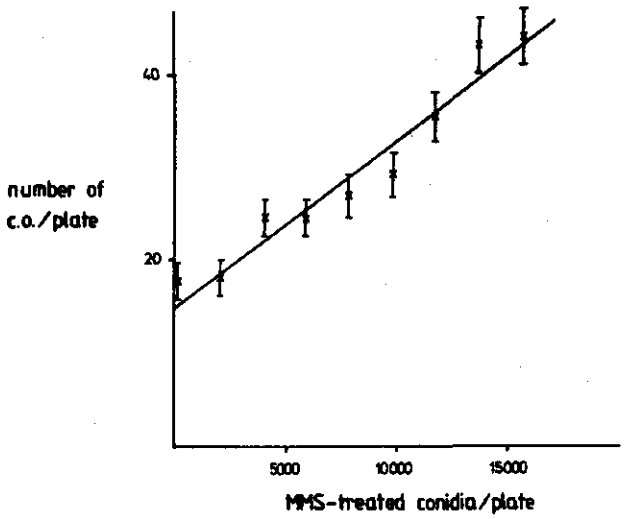


Legend: ○ average number of crossing-overs, after treatment with MMS
× average number of crossing-overs, untreated conidia

Analogous to the suppressor adenine system, the frequency of the crossing-over products found with the sorbose resistance system depended on the number of conidia inoculated per plate (fig 7). So here, too, the crossing-over frequencies can only be compared when they result from selection plates inoculated with a conidial concentration within a certain range (8,000 - 13,000 conidia / plate).

In an experiment in which mixtures of 2 conidial suspensions (untreated and MMS-treated, incubation method 2) were inoculated on SM + ace + SOR, no significant deviation of the linear relationship between the crossing-over frequency and the number of treated conidia per plate was found (fig 8). Here, the total number of conidia inoculated per plate was kept (nearly) constant, and only the ratio treated / untreated conidia varied.

Fig 8 Average number of sorbose resistant crossing-over products found in dependence of the number of MMS -treated conidia of strain D4 inoculated per plate SM + ace + SOR. A mixture of treated and untreated conidia was used (incubation method 2) in which the total number of conidia was kept (nearly) constant and the ratio treated/untreated conidia varied.



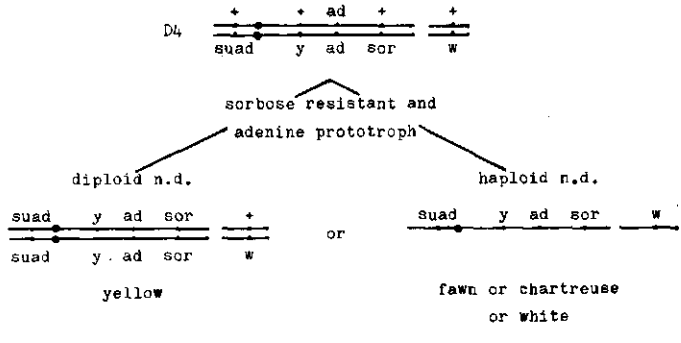
Legend: x average number of crossing-over products

Some experiments were performed (incubation method 2) in which the sorbose resistant recombinants were scored. And the accumulated results from the controls gave a spontaneous frequency of 6.6 yellow colonies (crossing-over and diploid non-disjunction products together), 0.46 green crossing-over products and 0.37 haploids per 10^4 conidia. The number of haploids was certainly underestimated, since haploids carrying the facA303 allele do not grow on acetate medium.

Since this selection system has no advantages over the other systems used and the crossing-over and non-disjunction products cannot readily be distinguished, this system has been discarded.

6.6 Combined selection of sorbose resistant and adenine prototroph recombinants.

The presence of the sorA allele and the suppressor adenine allele on chromosome I of strain D4 would offer an opportunity to select non-disjunction products on sorbose medium lacking adenine. Diploid non-disjunction products will be yellow and haploids fawn or chartreuse, or white. Schematically:



Apart from non-disjunction products, theoretically, products resulting from a crossing-over on both sides of the centromere can also be both sorbose resistant and adenine prototroph and will be found. But when only low concentrations of mutagen are used their frequency will be low.

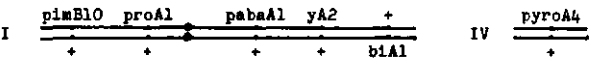
As a selective medium, MM + ace + paba, bio, ribo, thi, pyro, meth, cho and SOR (further on called SM + ace + SOR - ade) was used. Again the three plating procedures were compared: 1 the conidial suspension was plated on SM + ace + SOR - ade. 2 the conidial suspension was mixed with 5 ml SM + ace + SOR - ade (melted and cooled down to 50 °C) and poured out on a thin plate SM + ace + SOR - ade. 3 the conidial suspension was mixed with 5 ml SM + ace + SOR - ade (melted and cooled down to 50 °C) and poured into petridishes. When these plates had become solid, a second layer of ca. 15 ml SM + ace + SOR - ade was laid on the first. The incubation was for 3 - 4 days at 37 °C.

Procedure 1 and 2 were not at all applicable since no clear difference between sorbose resistant and sorbose sensitive colonies was seen. With procedure 3, after 4 days incubation at 37 °C, several colonies grew through the agar, some of which had yellow conidia but others were green or aconidiate. Generally, the conidial colour was faint. Upon isolation and analysis it was found that not all the colonies were adenine prototroph. Although the conditions for procedure 3 were varied (sorbose concentration in both layers, time interval between the pouring of the two layers), no satisfying test procedure could be found.

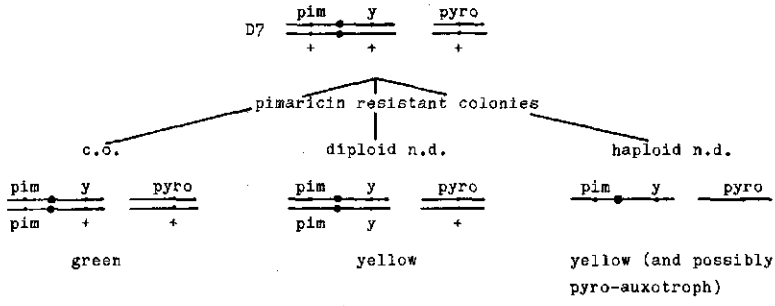
An attempt was made to give the non-disjunction products another selective advantage by constructing a diploid strain almost identical to strain D4 but carrying the *creA^{d}1* marker in trans-position to the *suad* allele (cross 216 x 473). *CreA^{d}* is a semi-dominant marker for a reduced growth rate and is located on the left arm of chromosome I (Arst & Cove, 1973). However, experiments with the new diploid strain did not reveal any advantage over strain D4 in testing for recombinant frequencies.

6.7 Selection of pimaricin resistant recombinants

De Bertoldi et al. (1980) developed a system in which the *pimB10* marker (resistance to pimaricin) of strain D7 is used to select mitotic crossing-over and non-disjunction products. Strain D7 has green conidia and is pimaricin sensitive. The genotype is:



A crossing-over between the pimB locus and the centromere gives a green pimaricin resistant colony, which can be proline auxotroph or prototroph. A diploid non-disjunction product will have yellow conidia and will be pro and paba auxotroph. Haploids can have the same conidial colour as the diploids arising after non-disjunction. Only when the conidial size is compared (using a Coulter Counter) to standard haploid and diploid strains can a differentiation be made between the haploid and diploid non-disjunction products. Schematically:



The selection medium consisted of MEA + glu + paba, pyro, pro, bio + 5.6 µM pimaricin, and the plates were incubated for 3 - 4 days at 37 °C. The recombinants were very easy to score and the recombinant frequency proved to be independent of the concentration of inoculated conidia. However, the recombinant frequency was found to be dependent of the pimaricin concentration used. As an example, the frequencies of recombinants found (recombinants / 10^4 colonies) after the plating of one conidial suspension on medium containing different pimaricin concentrations is given in table 43. From this empirical fact it follows that small variations in the pimaricin concentrations can influence

Table 43 Recombinant frequencies found after plating of the same (untreated) conidial suspension of strain D7 on different pimaricin concentrations.

pimaricin concentration	green colonies crossing-over freq.	s.d.	yellow colonies non-disjunction freq.	s.d.
(µM)	x 10^{-4}		x 10^{-4}	
1.9	no inhibition of growth of D7			
3.8	1.59	0.23	4.37	0.38
5.6	0.52	0.05	0.78	0.07
7.5	0.039	0.007	0.11	0.011
9.4	0.004		0.023	0.005
13.1	0		0	

the recombinant frequency; thus, the experiments have to be carried out very carefully.

It is also known that the incubation temperature is important and therefore must be kept constant (37 °C in our study) during selection procedures (van Tuyl, 1977).

Comparison of the conidial sizes showed that most of the yellow pimaricin resistant colonies (non-disjunctions) were haploid. Applying incubation method 4, in the control (growth on CM + glu) only 6 out of 201 yellow colonies tested have been found to be diploid. After growth on CM + glu + vinclozolin, no diploids were found among 205 yellow colonies and when grown in the presence of chloralhydrate, 5 diploids were found among 100 colonies. Not surprisingly so, since the sensitive allele is dominant.

Unfortunately, pimaricin itself is known to induce non-disjunctions. Interference of the residual presence of the chemical compound to be tested with the pimaricin activity might give false negative or positive results. However, when method 4 is used, this risk seems minimal.

6.8 Results obtained with the pimaricin resistance system

Only incubation method 4 has been used to test chemicals. The results are shown in the tables 44 - 51. Both the yellow (non-disjunction) and green (crossing-over) recombinants on each plate were counted. The information is given in essentially the same way as for the point mutations (see chapter 4.2).

Methylmethanesulfonate gave a large, significant increase of crossing-over products (table 44). The selectivity factor s was always about 1, or smaller than 1, and the mutagenicity factor m was much higher than 1. Unfortunately, too low numbers of recombinant conidia were added to the artificial suspension; thus the standard deviations are very high. The numbers of yellow colonies (non-disjunctions) were also significantly enhanced at the highest concentration. Although m was greater than 1, care has to be taken in interpreting these results since, in view of the high frequency of green recombinants due to a crossing-over, part of the yellow colonies can have resulted from a double crossing-over.

Nitrite, tested at the highest possible concentration, gave some

Table 44 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D7 on CM + glu + methylmethanesulfonate (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with recombinants are tested. Incubation for 3 days at 37 °C.

			crossing-over (green)								non-disjunction (yellow)							
			D7			D7 + enrichment					D7			D7 + enrichment				
exp.	conc. (mM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	428	0.53	0.11						821	1.0	0.2					
			399	0.78	0.16						725	1.4	0.3					
	0.59	(+)	1142	13 *	3						86	0.95	0.20					
2	0	+	53	1.3	0.7	20	7.2	1.5			199	5.0	1.0	86	31	6		
			60	2.0	0.4	106	8.6	1.8			195	6.5	1.3	554	45	9		
			77	1.7	0.3	106	7.5	1.5			197	4.2	0.9	589	42	7		
	0.29	+	74	22 *	5	571	25	5			5	1.5		263	11	2		
			1062	29 *	6	563	34	7	m=60	200	130	3.6	0.7	257	16	3	m= 1.5	0.5
			92	33 *	7	521	30	6	s= 0.3	0.9	93	2.4	0.5	246	14	3	s= 0.3	0.08
3	0	+	70	1.8	0.4	98	24	5			96	2.5	0.5	162	39	8		
			109	3.2	0.7	99	31	6			60	1.8	0.4	195	62	13		
	0.29	(+)	802	27 *	6	107	54	11	m=28	26	441	1.3	0.3	228	11	2	m= 3.9	1.4
			1094	41 *	9	140	38	8	s= 0.5	0.24	61	2.3	0.5	53	14	3	s= 0.2	0.06
	0.59	(+)	1334	36 *	7	174	58	12	m=19	14	80	2.1	0.4	234	7.1	1.5	m= 9.0	3.7
			1232	40 *	8	215	58	12	s= 0.8	0.5	73	2.4	0.5	352	8.6	1.8	s= 0.12	0.03
	1.17	(+)	1779	49 *	10	186	67	14	m=19	14	256	7.0 *	1.4	241	7.9	1.6	m=75	78
			1722	46 *	10	236	77	16	s= 1.0	0.6	229	6.1 *	1.3	305	9.1	1.9	s= 0.04	0.04

Legend: see table 40

Table 45 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D7 on CM + glu + sodium nitrite (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with recombinants are tested. Incubation for 4 days at 37 °C.

			crossing-over (green)							non-disjunction (yellow)						
			D7			D7 + enrichment				D7			D7 + enrichment			
exp.	conc. (mM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	67	1.7 e	0.4	131	45	9			134	3.4	0.7	181	63	13		
			149	6.5 e	1.3	144	36	7			78	3.4	0.7	285	72	15		
	1.8	+	259	6.9	1.4	308	93	19	m=0.8	0.3	394	1.0	0.2	36	1.0	0.2	g	
			313	8.2	1.7	295	85	18	s=2.2	0.6	347	0.78	0.16	30	0.8	0.2		
2	0	+	63	0.32e	0.07	378	4.4	0.9			112	0.57	0.12	82	11	2		
			35	0.12e	0.03	49	5.4	1.1			154	0.51	0.11	154	17	4		
			37	0.12e	0.03	49	5.2	1.1			78	0.25	0.05	131	14	3		
	1.8	+	178	0.49	0.10	59	5.3	1.1			31	0.09	0.02	31	0.25	0.05		
			149	0.35	0.07	75	7.4	1.5	m=1.7	0.5	27	0.06	0.01	34	0.30	0.06	m=10	3.4
			203	0.40	0.08	96	7.6	1.6	s=1.3	0.3	32	0.06	0.01	42	0.30	0.06	s= 0.02	0.03
	2.8	+	490	1.1 *	0.2	70	7.1	1.5			345	0.07	0.01	33	0.30	0.06		
			219	0.47	0.10	135	7.9	1.6	m=3.1	1.0	211	0.04	0.08	60	0.32	0.07	m= 6.4	2.2
						94	7.1	1.5	s=1.4	0.3				46	0.32	0.07	s= 0.02	0.03
3	0	+	160	3.9	0.8	44	59	12			154	3.8	0.8	168	226	47		
			79	2.9	0.6	52	74	15			111	4.1	0.8	218	311	64		
			119	6.0	1.2	80	108	22			142	7.1	1.5	259	350	72		
	1.37	+	195	4.5	0.9	38	52	11			34	0.78	0.16	88	11	2		
			95	2.5	0.5	48	62	13	m=1.3	0.4	21	0.56	0.12	94	11	2	m= 5	1.5
			159	6.6	1.4	63	84	17	s=0.8	0.2	31	1.3	0.3	86	10	2	s= 0.03	0.01
	1.8	+	228	4.0	0.8	93	88	18			188	0.30e	0.06	15	1.3	0.3		
			315	10	2	88	95	20	m=1.5	0.5	38	1.2 e	0.3	18	1.8	0.4	m=50	21
			327	9.6	2.0	135	122	25	s=1.2	0.3	44	1.3 e	0.3	37	3.0	0.6	s= 0.001	

Legend: see table 40

Table 46 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D7 on CM + glu + sodium azide (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with recombinants are tested. Incubation for 3 or 4 days at 37 °C.

			crossing-over (green)								non-disjunction (yellow)							
			D7			D7 + enrichment					D7			D7 + enrichment				
exp.	conc. (μM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	26	0.63	0.13						79	1.9	0.4					
(3 days)			22	0.52	0.11						53	1.2	0.3					
	4.7	(+)	17	0.46	0.11						47	1.3	0.3					
			14	0.32	0.09						60	1.2	0.3					
	19	(+)	3	0.05							13	0.24	0.07					
			2	0.08							5	0.2						
	28	(-)	16	0.18	0.05						50	0.56	0.12					
			21	0.27	0.06						53	0.67	0.14					
2	0	+	63	0.32e	0.07	378	4.4	0.9			112	0.57	0.12	82	11	2		
(4 days)			35	0.12e	0.03	49	5.4	1.1			154	0.51	0.11	154	17	4		
			37	0.12e	0.03	49	5.2	1.1			78	0.25	0.05	131	14	3		
	77	(+)	65	0.14	0.03	250	34 e	7			134	0.29	0.06	301	41 e	8		
			84	0.20	0.04	142	9.2e	1.9	m=0.21	0.06	177	0.42	0.09	233	15 e	3	m=0.4	0.1
			93	0.13	0.03	302	16 e	3	s=4.1	0.8	212	0.29	0.06	325	17 e	4	s=1.8	0.4
	96	(+)	54	0.15	0.03	243	22	5			120	0.34	0.07	371	34	7		
			71	0.16	0.03	222	20	4	m=0.22	0.07	231	0.51	0.11	543	50	10	m=0.28	0.08
			158	0.26	0.05	345	24	5	s=4.5	0.9	188	0.31	0.06	619	43	9	s=3.1	0.7
3	0	+	160	3.9	0.8	44	59	12			154	3.8	0.8	168	226	47		
(4 days)			79	2.9	0.6	52	74	15			111	4.1	0.8	218	311	64		
			119	6.0	1.2	80	108	22			142	7.1	1.5	259	350	72		
	77	+	111	1.7 e	0.4	90	462	95			86	1.3	0.3	97	467	96		
			169	3.6 e	0.8	80	404	83	m=0.16	0.05	81	1.7	0.4	47	223	46	m=0.26	0.08
			271	5.7 e	1.2	77	389	80	s=5.5	1.1	106	2.2	0.5	92	465	96	s=1.3	0.3
	96	(+)	87	1.7 e	0.3	99	110	23			68	1.3	0.3	112	124	26		
			167	3.4 e	0.7	247	174	36	m=0.45	0.14	60	1.2	0.3	296	208	43	m=0.67	0.2
			78	5.3 e	1.1				s=1.8	0.4	105	3.1	0.7				s=0.56	0.12

Legend: see table 40

Table 47 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D7 on CM + glu + acriflavine (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with recombinants are tested. Incubation for 3 or at 37 °C.

			crossing-over (green)								non-disjunction (yellow)							
			D7			D7 + enrichment					D7			D7 + enrichment				
exp.	conc. (μM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	8	0.07	0.02						49	0.41	0.10	204	15 e	4		
			15	0.15	0.04						71	0.71	0.17	311	42 e	10		
	9.1	+	9	0.09	0.03						29	0.29e	0.07	39	5.3	1.3	m= 4.9	1.9
			7	0.20							30	0.89e	0.21	43	8.3	2.0	s= 0.2	0.06
	18	(-)	13	0.20	0.06						36	0.61	0.14	102	17	4	m= 1.7	0.7
														138	21	5	s= 0.7	0.2
	23	(-)	6	0.14e	0.06						82	1.9 *	0.4	319	49	12	m= 3.1	1.2
			44	0.59*e	0.14						283	2.8 *	0.7	224	34	8	s= 1.4	0.4
2	0	+	62	1.5	0.3	57	48	10			112	2.7	0.6	291	247	51		
			64	1.6	0.3	56	52	11			116	2.8	0.6	158	146	30		
			60	2.0	0.4	25	30	6			95	3.2	0.7	138	163	34		
	9.1	(+)	77	3.7	0.8	107	119	25	m=0.7	0.2	125	6.0	1.2	148	164	34	m= 3.4	1.1
			82	2.3	0.5	140	104	21	s=2.6	0.6	241	6.7 *	1.4	112	83	17	s= 0.6	0.1
	18	(-)	37	1.7 e	0.4	64	80	16			212	9.9 *	2.0	633	790	160		
			108	5.9 *e	1.2	49	161	33	m=0.7	0.2	400	22 *	5	299	980	200	m= 1.6	0.5
			22	1.5 e	0.3	109	111	23	s=2.8	0.6	394	26 *	5	535	550	110	s= 4.1	0.9
	27	(-)	198	1.6 e	0.3	109	34 e	7			1395	122 *	25	226	1110	230		
			294	2.3 e	0.5	109	85 e	18	m=1.2	0.4	618	52 *	11	222	1710	350	m= 4.0	1.2
			31	4.5 *e	0.9				s=1.4	0.3	532	77 *	16				s= 7.3	1.7
3	0	+	19	0.08	0.02	43	3.8	0.8			65	0.28e	0.06	39	38	8		
			61	0.13	0.03	30	5.1	1.1			408	0.82e	0.17	162	28	6		
	18	(-)	152	0.37*	0.08	55	5.5	1.1	m=2.2	0.7	80	2.2 *	0.5	59	5.9e	1.2	m=19	7
			132	0.47*	0.10	88	11	2	s=1.8	0.4	79	3.1 *	0.6	77	16 e	3	s= 0.26	0.07
	27	(-)	85	0.35*	0.07	79	9.8	2.0	m=2.1	0.7	147	6.7 *	1.4	317	39	8	m=11	4
			38	0.33*	0.07	26	4.4	0.9	s=1.6	0.4	490	4.2 *	0.9	182	31	6	s= 0.9	0.2

Legend: see table 40

Table 48 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D7 on CM + glu + carbendazim (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with recombinants were tested. Incubation for 3 days at 37 °C.

			crossing-over (green)			non-disjunction (yellow)							
			D7			D7			D7 + enrichment				
exp.	conc. (µM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d	no.	freq.	s.d.	m or s	s.d.
1	0	+	292	0.81	0.17	678	1.9	0.4					
in 0.2%			331	1.0	0.2	498	1.5	0.3					
DMSO			262	0.56	0.12	579	1.2	0.3					
	0.65	+	573	1.4	0.3	911	4.2 *	0.9					
			413	1.2	0.3	1404	4.2 *	0.9					
	1.3	(+)	214	3.0 *	0.6	1521	108 *	22					
			173	2.0	0.4	897	51 *	11					
			197	2.6 *	0.5	1044	69 *	14					
2	0	+	50	1.4	0.3	105	3.0	0.6	198	13	3		
in 0.2%			6	0.7		12	1.5	0.4	225	8.5	1.8		
DMSO			82	1.3	0.3	157	2.4	0.5	256	10	2		
	1.3	+	25	0.76	0.16	91	3.0	0.6	92	3.4e	0.7		
			46	1.6	0.3	174	6.0	1.2	140	14 e	3	m= 5.1	2.9
			27	1.4	0.3	64	3.4	0.7	124	3.9e	0.8	s= 0.4	0.6
	2.0	(-)	43	2.2	0.5	357	107 e*	23	424	134	28		
			48	2.0	0.4	395	97 e*	20	374	114	23	m= 9.0	7.0
			43	1.2	0.3	275	44 e*	9	481	97	20	s= 4.0	2.6
3	0	+	8	0.11	0.04	36	0.48	0.11	178	16	4		
in 0.15%			9	0.12	0.04	52	0.69	0.16	269	31	7		
DMSO	0.46	+	16	0.18	0.05	78	1.2	0.3	221	28	7	m= 1.6	0.6
			9	0.14	0.05	97	1.1	0.3	317	30	7	s= 1.2	0.3
	0.65	+	4	0.1		51	0.60	0.14	196	15 e	4	m= 0.8	0.3
			7	0.06		74	0.59	0.14	325	48 e	11	s= 1.4	0.4
	0.98	(+)	9	0.13	0.04	79	1.1	0.3	145	13	3	m= 2.5	1.1
									251	22	5	s= 0.7	0.12
	1.51	-	5	1.6 *		218	69 *	16	396	191	45	m=25	13
			21	3.4 *	0.8	385	63 *	15	281	142	33	s= 4.4	1.7

Legend: see table 40

increase in crossing-overs (table 45). Higher concentrations could not be tested since only few colonies grew on those plates CM + glu + NO2. The number of non-disjunction products (yellow) were strongly reduced in the presence of NO2 caused by a selective disadvantage, so the effect on m is questionable. Rare recombinant types with different selection properties may have disturbed the calculation of m.

When azide was added to the medium both crossing-over and non-disjunction products had a selective advantage, and m was not found to be enhanced (table 46).

In the presence of acriflavine both the frequency of the crossing-over and the non-disjunction products was found to be enhanced. The (weak) effect on the crossing-over products was mainly caused by selective advantage. Only in one experiment m was somewhat enhanced. The effect on the non-disjunction frequency was much greater and m was always enhanced. The values for s, however, varied greatly, although they always increased with increasing ACR concentrations (table 47).

Table 49 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D7 on CM + glu + vinclozolin (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with recombinants were tested. Incubation for 3 days at 37 °C.

			crossing-over (green)			non-disjunction (yellow)							
			D7			D7			D7 + enrichment				
exp.	conc. (μM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d	no.	freq.	s.d.	m or s	s.d.
1	0	+	292	0.81	0.17	678	1.9	0.4					
in 0.2%			331	1.0	0.2	498	1.5	0.3					
DMSO			262	0.56	0.12	579	1.2	0.3					
	1.7	+	166	2.3 *	0.5	509	7.1 *	1.5					
			182	2.4 *	0.5	656	8.5 *	1.8					
			625	7.1 *	1.5	557	30 *	6					
			731	9.0 *	1.9	625	39 *	8					
			586	7.6 *	1.6	538	35 *	7					
	7.0	(-)	562	7.0 *	1.4	634	40 *	8					
			795	9.5 *	2.0	1303	78 *	16					
			605	7.3 *	1.5	597	36 *	7					
2	0	+	53	1.3	0.3	199	5.0	1.0	86	31	6		
in 0.2%			60	2.0	0.4	195	6.5	1.3	554	45	9		
DMSO			77	1.7	0.4	197	4.2	0.9	589	42	9		
	5.2	(-)	63	2.2	0.5	325	11	2	165	142	29		
			40	3.1	0.6	184	15 *	3	168	85	17	m= 0.8	0.3
			35	3.2	0.7	108	10	2	172	105	22	s= 2.9	0.7
3	0	+	50	1.4	0.3	105	3.0	0.6	198	13	3		
in 0.2%			6	0.7		12	1.5	0.4	225	8.5	1.3		
DMSO			82	1.3	0.3	157	2.4	0.5	256	10	2		
	3.5	(-)	66	3.6 *	0.7	528	29 *	6	459	127	26		
			89	4.1 *	0.8	631	29 *	6	536	105	22	m= 1.2	0.4
			76	2.9 *	0.6	650	25 *	5	556	98	20	s=10	2.8
	7.0	(-)	62	3.7 *	0.8	492	29 *	6	204	66 e	14		
			51	2.0	0.4	452	17 *	4	270	129 e	27	m= 1.2	0.5
			96	3.0 *	0.6	529	17 *	4	289	50 e	10	s= 7.5	2.0

Legend: see table 40

When carbendazim was tested the number of crossing-over products was somewhat increased, but much more effect was seen on the non-disjunction products. The mean cause was the increase of m (table 48).

In the presence of vinclozolin the number of crossing-overs and non-disjunctions was increased, however, for the non-disjunction products it was proved that this only was caused by a selective advantage of the yellow colonies (table 49).

Chloralhydrate had a heavy effect on the number of non-disjunction products caused by the increase of m. The crossing-over frequencies were not or little enhanced (table 50).

When p-fluorophenylalanine was tested the number of crossing-over and non-disjunction products remained constant, but the calculation of m and s showed that in both cases s was much smaller and m much greater than 1 (table 51).

Table 50 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D7 on CM + glu + chloralhydrate (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with recombinants were tested. Incubation for 3 days at 37 °C.

			crossing-over (green)			non-disjunction (yellow)							
			D7			D7			D7 + enrichment				
exp.	conc. (mM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d	no.	freq.	s.d.	m or s	s.d.
1	0	+	53	1.3	0.3	199	5.0	1.0	86	31	6		
			60	2.0	0.4	195	6.5	1.3	554	45	9		
			77	1.7	0.4	197	4.2	0.9	589	42	9		
	6.0	(+)	17	1.5e	0.3	37	3.3	0.8	147	9.9	2.0		
			8	0.9e	0.6	50	5.6	1.2	17	5.1	1.1	m= 8.9	4.7
			5	0.4e		36	3.1	0.6	30	5.8	1.2	s= 0.09	0.04
	0	+	242	1.4	0.3	598	3.6	0.7	129	22	4		
			256	1.8	0.4	442	3.2	0.7	158	35	7		
			299	1.8	0.4	609	3.7	0.8	95	25	5		
			201	1.8	0.4	453	4.0	0.8	345	23	5		
			158	0.90	0.21	349	2.0	0.4	230	24	5		
			236	1.5	0.3	442	2.7	0.6					
	8.7	(-)	23	0.41	0.08	811	15 *e	3	225	38	8		
			49	0.65	0.13	814	11 e	2	217	32	7	m= 5.6	2.1
			43	0.93	0.19	1007	22 *e	5	189	28	6	s= 0.8	0.2
			32	0.39	0.08	709	8.7e	1.8	242	29	6		
3	0	n.s.				224	3.1	0.7	92	27	6		
						201	3.5	0.8	108	21	5		
						407	2.0	0.5	173	27	6		
	4.9	n.s.				106	58 *	14	358	122	29		
						204	49 *	12	174	96	23	m=16	12
						286	72 *	17	112	47	11	s= 1.3	0.8
4	0	+	83	0.59	0.12	229	1.6	0.3					
			65	0.56	0.12	200	1.7	0.4					
			105	1.1	0.2	284	3.0	0.6					
	2.4	+	76	0.77	0.16	369	3.7	0.8					
			146	1.6	0.3	500	5.5	1.1					
			104	1.2	0.3	492	5.9	1.2					
	4.8	(+)	71	0.79	0.16	401	4.5	0.9					
			69	0.97	0.20	392	5.5	1.1					
	9.7	-	60	1.0	0.2	386	6.7*	1.4					
			26	4.8 *	1.0	894	163 *	34					
			13	3.9 *	1.1	576	173 *	36					

Legend: see table 40

6.9 Preliminary conclusions

When it is not intended to distinguished between the different processes, the scoring of mitotic segregation is the most practical and the easiest selection procedure.

The suAladE20 allele can be used to select recombinants, but complications arise since there is a concentration effect.

The sorbose resistance allele can also be used, but is less suitable since the same concentration effect occurs and, beside that, the crossing-over products cannot be distinguished from diploid non-disjunction products.

Selection of adenine prototroph and sorbose resistant recombinants

Table 51 Frequency of crossing-overs and non-disjunctions (per 10^4 conidia) after growth of strain D7 on CM + glu + para-fluorophenylalanine (incubation method 4). Both normal conidial suspensions and suspensions artificially enriched with recombinants were tested. Incubation for 3 days at 37 °C.

			crossing-over (green)								non-disjunctions (yellow)							
			D7			D7 + enrichment					D7			D7 + enrichment				
exp.	conc. (µM)	coni-diation	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.	no.	freq.	s.d.	no.	freq.	s.d.	m or s	s.d.
1	0	+	4	0.4							14	0.13	0.03	219	18	4		
			11	0.2	0.06						17	0.20	0.06	106	9.4	1.9		
			8	0.2	0.07						13	0.28	0.08	91	20	4		
	55	+	7	0.06	0.02						54	0.49	0.10	61	4.5e	0.9		
			13	0.14	0.04						60	0.64	0.13	128	13 e	3	m=3.9	1.2
			3	0.15							73	0.82 *	0.17	214	22 e	5	s=0.8	0.2
	82	(+)	11	0.14	0.04						20	0.26	0.06	358	9.8	2.0		
			11	0.12	0.04						44	0.50	0.10	832	8.7	1.6	m=3.2	1.0
			15	0.21	0.06						41	0.58	0.12	100	17	3	s=0.7	0.2
2	0	+	19	0.08	0.02	43	3.8	0.8			65	0.28 e	0.06	39	38	8		
			61	0.13	0.03	30	5.1	1.1			408	0.82 e	0.17	162	28	6		
	96	(+)	20	0.08	0.02	17	1.7	0.4	m=2.0	0.9	180	0.68	0.14	167	17	4	m=2.6	1.0
									s=0.4	0.1							s=0.5	0.14
	109	(+)	41	0.20	0.04	18	1.9	0.4	m=3.6	1.3	216	1.0	0.2	118	13	3	m=4.6	1.6
			50	0.13	0.03	23	2.2	0.5	s=0.4	0.1	385	1.0	0.2	162	15	3	s=0.4	0.1
	136	(+)	65	0.16	0.03	19	1.8	0.4	m=4	1.5	30	0.83	0.17	107	9.9	2.0	m=6.0	2.1
			46	0.14	0.03	14	1.6	0.4	s=0.4	0.1	364	1.1	0.2	99	11	2	s=0.29	0.03
3	0	+	62	1.5	0.3	57	48	10			112	2.7	0.6	291	247	51		
			64	1.6	0.3	56	52	11			116	2.8	0.6	158	146	30		
			60	2.0	0.4	25	30	6			95	3.2	0.7	138	163	34		
	96	(+)	205	0.39	0.08	43	1.9e	0.4	m=2.3	0.8	125	2.6	0.5	104	50	10	m=3.0	1.0
			109	0.27	0.06	67	5.8e	1.2	s=0.08	0.02	73	3.0	0.6	76	72	15	s=0.32	0.07
	178	(-)	205	0.45	0.09	97	12	2			90	2.2	0.5	89	115	24		
			232	0.55	0.11	91	7.9	1.6	m=1.6	0.5	61	2.4	0.5	95	91	19	m=1.7	0.5
			206	0.72	0.15				s=0.22	0.05	53	3.0	0.6	52	83	17	s=0.5	0.1

Legend: see table 40

at the same time seems not possible, since several procedures were tested and were not successful.

Selection of pimaricin resistant recombinants with strain D7 is much less complicated and has therefore been preferred.

MMS strongly induces crossing-overs. The much weaker effect on the non-disjunction frequency may be an artefact, since double crossing -over products can have the same phenotype as the non-disjunction products, both with strain D4 using the suppressor adenine system and with strain D7 using the pimaricin resistance system.

VIN has only little or no effect on strain D4, but with strain D7 the frequencies of the crossing-overs and the non-disjunctions were enhanced. At least for the non-disjunction frequency it was proved that this was caused by selective advantage of the recombinants.

CH does not or only weakly have an effect on the crossing-over frequency but non-disjunctions are strongly induced, both in strain D4 and D7.

NO2 gives some crossing-overs in strain D7, but to measure the non -disjunction frequency was not possible because of the strong selective disadvantage of the recombinants.

N3 does not induce crossing-overs and non-disjunctions in strain D7.

ACR seems to induce crossing-overs in strain D7 , but the effect is not significant. Non-disjunctions are induced (significantly).

MBC induces non-disjunctions in strain D7. The crossing-over frequency is also enhanced, but it has not been checked whether this was caused by selection or induction.

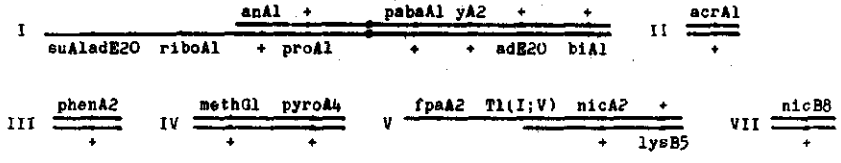
FPA induces both crossing-overs and non-disjunctions (obscured by a selective disadvantage) in strain D7.

7 RECESSIVE LETHAL DAMAGE

The ultimate effect of a chemical compound on the genetic material is cell-death. But, since lethality can also occur after interaction of the compound with other cell components, this cannot be taken as evidence for genetic activity of the chemical compound.

However, if it can be proved that a lethal effect is induced which does not act on the target cell itself but on the progeny of this cell, then the chemical compound must have interacted with the genetic material. In this case, on one of the chromosomes a vital gene function is disturbed by a point mutation, or a part of a chromosome is lost by a deletion, or a translocation has yielded a non-viable combination.

In this study strain D3, developed by Morpurgo et al. (1978), has been used since this is the easiest system for measuring recessive lethals in Aspergillus (see chapter 1.2). The genotype of strain D3 is:



Using strain D3, 3 of the 16 chromosomes can easily be surveyed for the presence of recessive lethals, for when this diploid is forced to haploidize on medium supplemented with FPA, only those haploids can grow which carry the fpaA2 allele for resistance to FPA. FPA has two functions in this method: 1 induction of haploids and 2 selection for FPA resistant segregants. And, since the fpaA gene is located on the translocated fragment, a FPA resistant haploid must carry the corresponding homologue of chromosome I. In addition, haploids carrying the phenA2 allele cannot grow on FPA medium, so the other homologue must be present in the haploids found. Thus a haploid found must have the following genotype:

I anA1 pabaA1 yA2 III phenA2 V fpaA2 T1(I;V) nicA2

In all cases haploid sectors will have a yellow conidial colour. This implies that when on one of the chromosomes a vital gene function is disturbed no sectors with yellow conidial colour are seen.

After treatment with a mutagen, the colonies were firstly grown on CM + glu + des, since dominant lethal damage must be excluded. Then these colonies were tested for recessive lethal damage by inoculating them on CM + glu + FPA (5 colonies / plate). After 7 days incubation at 37 °C, the percentage of colonies with yellow sectors was counted.

Complete medium was not found to be satisfactory. Up to 5% of the colonies grown from untreated conidia did not give sectors. Since the spontaneous frequency of recessive lethals is not likely to be as high as found here, not all colonies which can give sectors on FPA medium did so.

Modified Czapex Dox medium, in which the $NO3^-$ was replaced by $NH4^+$, supplemented with glucose, growth factors (no phenylalanine) and FPA gave better results, since the number of non-sectoring colonies in the controls was reduced to ca. 2%. The zero level has not been reached. After addition of neopeptone (2 g / l) to this medium, the conidiation was improved, but the number of non-sectoring colonies in the controls did not change.

The results of one experiment, in which the number of recessive lethals was measured after treatment of conidia of strain D3 for 1 hour in liquid suspension (incubation method 2), are shown in table 52. Both MMS and BED have been found to enhance the number of recessive lethals. But only with BED treatment the increase was found to be significant.

Table 52 Recessive lethals found after treatment of conidia of strain D3 with MMS and BED for 1 hr in liquid suspension (incubation method 2). Firstly conidia were grown on CM + glu + des and then they were inoculated on modified Czapex Dox medium + glu + growth factors and amino acids + FPA. After 7 days incubation at 37 °C, the number of colonies without yellow sectors were counted.

mutagen	conc (mM)	surv. %	no. of colonies tested	no. of colonies without sectors	recessive lethals %
none	0	100	99	2	2
MMS	11.7	98	77	9	12
MMS	23.4	57	75	9	12
BED	9.7	36	65	9	14 *
BED	19.3	7	71	18	25 *

Legend: surv. survival
* significant at P<0.05, one-sided, when a Poisson distribution is assumed.

In another experiment replicas were made from platings on FPA medium, and it was observed that most non-sectoring colonies in the controls gave sectors in the replicas (table 53). Therefore, not all of the colonies which can give sectors do indeed so, or mixed colonies exist in which part of the conidia carry a recessive lethal and the other conidia are normal. This implies that large amounts of colonies have to be tested when a weak mutagenic activity is to be recognized as significant.

Table 53 Recessive lethals found after treatment of conidia of strain D3 with different concentrations of NMS (in liquid suspension for 1 hr, incubation method 2). For the procedure see table 52. Every colony was tested twice.

conc. (mM)	surv. %	no. of colonies tested	different results in the two platings	no sectors found in the two platings
0	100	50	2	0
11.7	39	50	1	3
17.6	39	35	1	3
23.6	6	49	1	10 *

Legend: see table 52

8 GENERAL DISCUSSION

8.1 Incubation methods

8.1.1 Method 1

The plate incorporation assay (method 1) is the most simple incubation method for mutagenicity testing. Here the test strain is incubated directly on plates containing the chemical compound to be tested. These plates are then screened for mutants carrying biochemical, morphological and / or colour markers. With Aspergillus as a test organism, this method - with varying conditions - has been applied successfully in screening for point mutations (Bignami et al., 1982), loss of a duplicated fragment (Roper et al., 1972), crossing-overs and non-disjunctions (Kappas et al., 1974). Although it is the most simple way of testing chemicals, complications may arise when the compound to be tested is toxic or affects conidiation. Moreover, since the survival is not known, exact quantitative data cannot be obtained, unless only few colonies are tested per plate. The difference in fitness between the mutants / recombinants and the original strain can possibly be different on media with and without the chemical to be tested, resulting in an over- or underestimation of the mutagenic / recombinogenic activity. This may be the case with our only experiment using method 1 (see 6.2), where among others the sectoring of strain D4 after growth on media with and without vinclozolin was compared. Under somewhat different conditions (using method 4, see chapter 6.4) such a difference in fitness has indeed been observed.

Although this method is valuable as a pre-screening test, we made only limited use of it, since quantitative data were preferred.

8.1.2 Method 2

When conidia are tested in liquid suspension (method 2) prior to plating on selective or complete medium, quantitative data can more easily be obtained. Toxic effects of the chemical will be recognized since the survival of the conidia is measured as a routine. The

inhibition of growth on selective or complete medium will be much less than with method 1, for the chemical is removed by filtration and subsequent washing of the conidia.

With incubation method 2 a number of possible complicating aspects should be kept in mind:

1 Some inhibition of growth is still observed after mutagen (MMS) treatment, sufficient to complicate the scoring of the suad recombinants (6.3). This is perhaps caused by the residual presence of the mutagen on the surface of, or inside the conidia, or more likely by physiological damage to the conidia. The effect was pronounced after azide treatment: the surviving conidia hardly grew on acetate as a carbon source, but did grow when arabinose or glucose was used (5.8).

2 For practical reasons, CM + glu + des plates have been used for the viability counts. Many mutant types can grow on this medium, but cannot grow on MM or MEA, the basic media for the selection plates. Therefore it is possible that not all conidia scored as viable on CM, are also viable on the selection plates. The difference, however, will be very small. Only when a very potent mutagen is tested its mutagenic action will be somewhat underestimated. The case of azide, discussed before, is an exception.

3 Often considerable variation is seen between the results of the repeated experiments with a given mutagen. These experiments were (almost) all started from different colonies of the test strain. Differences in the condition of these colonies or differences in the mutation / recombination rate during first growth can have occurred and may have led to the observed variation. Therefore, when the spontaneous frequency in a particular experiment was found to be much higher than usual, this experiment was discarded.

4 The existence of a germination inhibitor which might influence the reproducibility of mutagenicity testing has been reported by Scott et al. (1972). Therefore, in part of our experiments special care was taken to remove this inhibitor. However, no consistent effect of this procedure has been found in these experiments. As shown in the tables 2 (MMS/110) and 25 (BED/007), the experiments carried out without removal of the germination inhibitor gave a somewhat lower survival (in accordance with Scott et al., 1972), but in the tables 5 (BED/110 and 470), 7 (ACR/110) and 24 (MMS/007) no clear difference is seen. In table 28 (ACR/007) even the opposite effect is found: a lower survival

after removal of the germination inhibitor. The reproducibility is also not clearly improved by removal of the germination inhibitor. Thus, we did not notice any advantage of the procedure. The inhibitor has possibly been automatically removed by our method.

5 For the statistical calculations, a binomial distribution of the mutant or recombinant frequencies is assumed. However, in doing so only the random variation in the mutant or recombinant numbers is accounted for, but not the variation in the numbers of viable conidia. Therefore, care must be taken to assure that, compared to the former, the latter variation is low (in relation to the actual value found). This will only be true when low numbers of mutants or recombinants are counted, since then (on the basis of the binomial distribution) their variation will be relatively high. In the present study often more than the maximum number of mutants or recombinants allowed by the formula of Katz (1979) have been counted, and in these cases the standard deviation was estimated assuming that the maximum number had been counted. In doing so, the real standard deviation has been overestimated. In future studies the standard deviation has to be calculated more effectively by using more plates for measuring the survival.

An advantage of method 2 is that two effects of a mutagen can be compared by regression analysis: mutation or recombination frequency and survival. The slope of the regression line can be used to compare the effects of different mutagens.

From our experiments we conclude that the liquid suspension test (method 2) can be readily used for measuring the mutagenicity and recombinogenicity of agents acting on dormant conidia.

8.1.3 Method 3

Since a number of mutagens, e.g. those which induce non-disjunctions, preferably or exclusively act on dividing cells, methods have been developed to use germinating conidia of Aspergillus for mutagenicity and recombinogenicity testing. The incubation in liquid MM, containing only 0.2% agar (method 3), has been used successfully by others (de Bertoldi et al., 1980; Gualandi et al., 1979). However, in the present study this method has not been successful, since in spite

of the presence of the agar many conidia were lost during prolonged incubation (fig 2). Some of the conidia germinated early and probably gave rise to the big clumps observed later on. Our results are also at variance with the findings of Bainbridge (1971) who reported the appearance of the first germ tubes after 3 hours incubation. In our study germination of the conidia was obviously not synchronous, and some of the conidia were not in the dormant stage, perhaps due to our way of preparing the conidial suspensions. In general, the use of a long incubation period in liquid medium with a filamentous fungus may not be optimal.

In the two experiments we performed with method 3, the strains 110 and 007 were used to test 9-aminoacridine (tables 9 and 30). No effect was observed and the survival data were rather peculiar, probably caused by differences in the clumping of the conidia both in the presence and absence of 9AA.

We found incubation method 3 to be unsatisfactory.

8.1.4 Method 4

The media mediated assay, our method 4, has been used for the testing of germinating conidia. Here the chemical to be tested is incorporated into a plate CM + glu on which conidia of the test strain are grown. Afterwards, a conidial suspension is made and analysed for the mutant / recombinant frequency. With this method the chemical is present during all mitotic cell stages. The actual concentration of the compound will more or less decrease during incubation, depending on its stability, but on the other hand the metabolites are automatically included in the test. The pH of the plates rises during incubation from 4.5 or 6.0 to 7.6. Of course, the mutations or recombinations which take place early in mycelial growth will have the most impact on the resulting mutant or recombinant frequency.

Some remarks can be made about incubation method 4:

1 Compared to method 2, method 4 includes a longer growth period which adds an extra opportunity for spontaneous mutations or recombinations resulting in a (generally) higher spontaneous mutation or recombination frequency.

2 The variation between the repetitions within the same experiment is often high and the differences sometimes significant. In some cases

this variation can be explained. On the mutagen containing plates random variation in the mutant or recombinant frequency occurs within the limits of the Poisson distribution. But when at the same time a selective advantage for the mutants or recombinants exist, these variations will become greater. When strain D7 is used a slight variation in the pimaricin concentration can already influence the number of recombinants counted, as was shown in chapter 6.7.
2 The toxicity of a compound cannot be easily quantified with method 4, since conidiation can only be classified visually. This classification in inaccurate, and since in our study there sometimes was a large time interval between the repetitions of an experiment, some of the variation in the classification can be explained this way. Another kind of toxic effect was found when the highest concentrations 9-aminoacridine were tested: an unexpectedly high percentage of conidia did not germinate, as was concluded from the large difference between the results of the platings on CM + glu + des and the Coulter Counter data (data not shown).

The method was improved by including a control to distinguish between a real mutagenic or recombinogenic activity and a selective advantage in the presence of the chemical compound. To this end we also tested a second conidial suspension, artificially enriched with mutant conidia. A formula is given (see chapter 3.4) to calculate the relative contribution of m (mutagenicity factor) and s (selectivity factor) to the enhancement of the mutant frequency. In this way a mutagenic or recombinogenic activity is found for some chemicals, and a selective advantage for the others.

The proposed model for calculating m and s includes two assumptions which are not entirely valid:
1 The mutant and recombinant frequencies found after growth in the presence of the chemical to be tested are all compared with the frequencies after growth on CM + glu without the chemical. It was found (data not shown) that on CM + glu a selection or suppression of the mutants or recombinants has taken place. So all values of m and s are only relative ones.
2 Not all mutants or recombinants of the same type will have the same fitness. To cope with this problem, about 20 isolates were mixed and this mixture was used to make the artificial conidial suspension.

However, the natural ditribution of the mutant or recombinant types will not be precisely copied, so there remains a difference between the natural situation and the simulation. When an extreme selective disadvantage is found (as in table 34), a positive value of m must be interpreted with caution. The reason is that a rare mutant or recombinant type, not added to the artificially enriched suspension, may have had a wildtype-like growth rate in the presence of the chemical. In this case, an enhanced m value would not reflect a mutagenic or recombinogenic effect.

The standard deviations of m and s are often very high. They are minimal when the difference between the spontaneous mutation or recombination frequency of the normal suspension and that of the artificial suspension are large. However, this difference should not be too extreme, for then the scoring of the mutants or recombinants no longer takes place under comparable conditions. In our study it was found to be most practical when the spontaneous mutant or recombinant frequency in the artificial suspension is 10 to 20 times the spontaneous frequency in the normal suspension.

Our conclusion is that the media mediated assay is most suitable when germinating conidia and growing mycelium are to be tested. However, an additional control experiment has to be done in order to distinguish mutagenicity from selection processes.

8.2 Test procedures

8.2.1 Point mutation

For measuring the point mutation frequency, the methionine system has been used. Strains 110 and 470, both carrying the methG1 allele, are likely to react in the same way. In one experiment the two strains have been used to measure the mutagenicity of the same compound (BED, table 5). Identical results were obtained. However, strain 110 is preferred to strain 470 since it produces more conidia.

Three mutant classes (A, B and C, each containing mutants at probably 2 loci) can be distinguished. Thus, when the genes concerned differ in mutability, resulting in different mutation frequencies for the three classes, such a specific action of the mutagen can in principle be discovered. In the present study the distribution of the

mutants over the three classes is known for method 2 only (tables 2-8). NO2 gives clearcut results: predominantly mutants of the classes A and C are induced. These results, however, are not conform the data shown in the review of Scott et al. (1982), where as many B as C type mutants are reported. This discrepancy is not likely caused by a misclassification, since type B mutants can easily be distinguished from other mutant types. The other chemicals did not give such clearcut results: MMS induces about equal amounts of mutants of all three classes. ACR gives only low numbers of type C mutants, compared to A and B, and so does BED. N3 gives varying results in the different experiments.

If a specific action of a mutagen has to be shown, the 2-thioxanthine system is more suitable. For in this system mutants at 8 loci can be scored, which can all be distinguished by growth and complementation tests.

In all other cases the methionine deficient strain 110 is very suitable for measuring the point mutation frequency.

8.2.2 Loss of a duplication fragment

The instability of duplication strains can readily be used for testing chemicals. The process involved in this instability is not known, however. Repair mechanisms acting on chromosome lesions seem to play a role, for certain chemicals which are known to inhibit the repair enzymes increase the instability (Roper et al., 1972). The presence of the duplication provokes its own instability. Random breaks are not likely since often, but not always, a (nearly) exact restoration of the wildtype is obtained. One hypothesis states the presence of identical base pairs at the terminal ends of each chromosome, and an illegitimate cross-over between these ends of different chromosomes might be possible. When the duplicated fragment is connected distal to these base pairs, this fragment may be lost by such a crossing-over. This model may be valid for some systems (Newmeyer & Galeazzi, 1977), but cannot explain the occurrence of sectors with a morphology which is intermediate between the original duplication strain and wildtype strains, as found for Dp (I→II) studied here.

When the yellow marker is used to select segregants from the (I→II) duplication some segregants are recovered too, which arise after a

normal crossing-over between the homologous chromosome fragments (see chapter 5.6). This can only be avoided when special care is taken: incubating only few colonies per plate and scoring only sectors with an improved morphology (as was done by Nga & Roper, 1968), or isolating the yellow sectors and compairing their morphology to standard strains.

If this is not done (as is the case in the present study and in other studies, e.g. Normansell & Holt, 1979), also a few crossing-over products may be included among the colonies scored as "deletions".

The introduction of the sorA allele and the scoring of the segregants on sorbose containing medium has two effects: (1) all colonies are small but (2) the sorbose resistant colonies are the largest and their conidial colour is brighter. Plating up to 100,000 colonies per plate is possible. The frequency of yellow colonies is lower than after plating on CM + glu. Such a difference is to be expected, for the colonies are much smaller on sorbose medium and only those yellow colonies or sectors are scored which arise very early in colony development. On CM + glu, the yellow sectors which arise during mycelial growth are counted as well. It is also possible that part of the yellow sectors counted on CM + glu arise after an interstitial deletion which does not cover the sorA region (the meiotic map distance between yA and sorA is about 33 units, see 5.4). Nga and Roper (1968) proposed the occurrence of such interstitial deletions as an explanation for a series of sectors with an improved but not yet wildtype morphology. In their detailed study, however, the occurrence of crossing-overs has not been considered. The improved morphology must have been caused by a deletion of (part of) the duplicated fragment. But the sectors which still have a crinkled morphology are not necessarily caused by a deletion since crossing-overs (which in the present study are shown to occur, see 5.6) can also explain the phenotype of these sectors.

The construction of a duplication strain heterozygous for the sorA allele (strain 007) reduces the labour of measuring the instability of duplication strains.

8.2.3 Crossing-over and non-disjunction

Several procedures for measuring the crossing-over and non-disjunction

frequency have been investigated. When no distinction between these two processes is required, the easiest way is to score the segregation of colour markers. Isolation of the sectors and subsequent testing for biochemical markers can be undertaken to elucidate the inducing mechanism when suitable markers are available, but this technique is laborious. It seems possible that with this method a selective advantage of (one of) the recombinant types can give false positive results. Small patches with recombinant conidial colour, which remain undiscovered in the control plates, may grow out to give visible sectors in the presence of the chemical to be tested if there is a selective advantage for these recombinants. This may be the case with vinclozolin, which induces an enhanced sectoring of strain D4, for when tested with incubation method 4, the only effect was a selective advantage (tables 39 and 49).

If it is intended to distinguish between the processes which can lead to sectors with non-parental phenotype, it is less laborious to select special recombinant types. Theoretically, the suAadE allele should be very suitable, for the crossing-over products and the diploid and haploid non-disjunction products can directly be distinguished by their conidial colour (green, yellow and white / chartreuse / fawn, respectively). In practice this system is less suitable, for the colour of the recombinants is rather faint and a conidial concentration effect is observed (see fig 6). This is caused by the leaky growth of the unchanged test strain on medium without adenine, as the number of mitotic cell divisions, and therewith the chance of recombinants, depends on the number of conidia grown per plate. When only the larger colonies are counted this concentration effect is reduced. But the delayed germination of the conidia after mutagen treatment (method 2), (also observed on the CM + glu + des plates used for the viability count) interferes with this correction. In addition, the colony size of the non-disjunction products is generally found to be smaller than the size of the green cross-over colonies. This can be expected as the non-disjunction products arise from aneuploids which have a reduced growth rate. When incubation method 4 is used, the fysiological effects of the chemicals on the conidia are much smaller and consequently the scoring of the recombinants is much simpler.

With incubation method 2 the observed numbers of crossing-overs are somewhat lower than estimated by Käfer (1977). Also hardly more haploid

than diploid non-disjunction products are counted. The difference was expected to be much greater since adenine prototroph haploids can arise after a non-disjunction of any of the chromosome pairs, whereas only a non-disjunction of the first chromosome pair can lead to adenine prototroph diploids. Probably not all haploids are recovered as a result of markers on other chromosomes which give them a slower growth rate.

Another possibility for counting recombinants is to select sorbose resistant colonies from a diploid heterozygous for the sorA allele. However, here the diploid non-disjunction and crossing-over products cannot be visually distinguished. A similar conidial concentration effect as with the suad system is observed (fig 7). When a mixture of treated and untreated conidia is plated and the total conidial concentration is kept constant, a linear relationship between the number of treated conidia and the number of recombinants is observed (fig 8). This indicates that only data obtained from experiments in which approximately the same number of conidia were plated on one petridish can be compared. The scoring of the recombinant sorbose resistant colonies from strain D4 is more complicated than the scoring of the sorbose resistant segregants of strain 007. Simple platings on sorbose medium are not suitable since too much variation in growth rate and conidiation of the recombinants, especially the non-disjunction products, is observed. Therefore the conidia have to be mixed through the agar.

Since the sorA system bears no advantages over the other available systems, we dropped it for further studies.

A combined selection for adenine prototroph and sorbose resistant colonies did not work. Theoretically, only non-disjunction products can grow. Several experimental procedures were tried out, but none was satisfactory. The main problems were the faint conidial colour and the fact that not all growing colonies were adenine prototroph. The introduction of the $creA^{d}$ allele (semi-dominant for a reduced growth rate) in trans-position to the sorA and suAadE markers did not improve the scoring of the non-disjunction products.

When the pimB allele for resistance to pimaricin, present in strain D7, is used to score recombinants no background growth is seen. Non-disjunction and crossing-over products can be directly distinguished by their conidial colour (yellow and green, respectively), but haploid and diploid non-disjunction products have the same conidial colour and can only be distinguished when conidial size is compared.

The recombination frequency is independent of the conidial concentration and a high number of recombinants (up to 200) can be scored per plate. However, the number of recombinants depends strongly on the pimaricin concentration (see 6.7). The yellow colonies are predominantly haploid, as expected (see 1.2).

Pimaricin is reported to induce non-disjunctions itself. Therefore a synergistic interaction of the chemical to be tested and the pimaricin can give false positive results. In case of doubt, another system must be used to verify the results. When incubation method 4 is used the risk of occurrence of false positive results seems minimal.

Other procedures for measuring the frequency of mitotic crossing-overs and non-disjunctions are available, but have not been used in our study. Selection of FPA resistant recombinants from a diploid strain heterozygous for the FPA resistance allele, has been used successfully by others (Bignami et al., 1974; Morpurgo et al., 1979). Positive effects on the non-disjunction frequency, however, must be verified by another system since FPA is known to induce non-disjunctions itself.

It is also possible to plate, after mutagen treatment, the conidia in low concentration on CM + glu and to score all colonies with recombinant colour and all abnormal colonies. Isolation and genetic analysis can reveal the inducing process (Song & Kang, 1979; Bellincampi et al., 1980). In this way a detailed analysis is possible, though laborious.

Our conclusion is that the crossing-over and non-disjunction frequencies can be estimated by observing mitotic segregation. But when the two processes are to be distinguished, selective systems are preferred. Selection of pimaricin resistant recombinants proved to be the most suitable system, in combination with the media mediated assay (incubation method 4).

8.2.4 Recessive lethal damage

Recessive lethal damage is another kind of genetic damage which occurs in diploids. When this is to be measured, the procedure of Morpurgo et al. (1978) is the simplest one. However, one replating is still needed to distinguish between recessive and dominant lethals. So only a limited number of colonies can be tested. As a consequence, the increase over the spontaneous frequency must be large to find a significant difference. Therefore, the number of non-sectoring colonies in the controls must be reduced to a minimum. However, in all cases over 2% of the colonies did not give sectors when untreated colonies were plated (on sector inducing FPA medium), even when the most optimal conditions were chosen. It is not likely that the real spontaneous frequency is as high as that (Morpurgo et al., 1978, reported a zero level). In one experiment it was shown that the replicas of the non-sectoring colonies in the control gave sectors. It is possible that mixed colonies exist when a lethal is induced in only one of the two chromatids, but in the case of the control it is more likely that not all colonies which can give sectors did so.

Therefore, too many colonies have to be tested when a weak mutagenic activity is to be recognised, so the method will be too laborious for routine use. Nevertheless, the method still has its value for special investigations.

8.3 Chemical induction of mutants and recombinants

The results of all experiments are summarized in table 54. Two alkylating agents have been examined: methylmethanesulfonate (MMS) and butadienediepoxide (BED). Alkylating agents induce mainly point mutations, but also other kinds of genetic damage by alkylation of the genetic material. The main alkylation product is generally found to be N^7-alkylguanine. However, investigations in which the mutagenic and carcinogenic effects of several alkylating agents have been compared, show that these effects are better correlated with the O^6-alkylguanine concentration. But also errors inserted during repair of damage to the DNA can lead to the expression of mutants (Pegg, 1977).

MMS has been shown to induce e.g. point mutations in Salmonella, micronuclei and sperm abnormalities in mice, where it is also

Table 54 Summarized results

strain	mutation type	incubation method	chemical compound: MMS	BED	NO2	N3	9AA in H_2O	9AA in DMSO	ACR	FPA	CH	MBC	VIN	see table
110	point mutations	2	+++	+++	+++	+++		o	+					2 - 8
		3						o						9
		4: total	+++	+++	o	o	++	o	o			o	o	10 - 17
		m	+	+	o	o	+		o			o		
		s	+	-	+	-	o		+			-		
007	deletions or crossing-overs	2	++	++	+	+		o	+					24 - 29
		3					o	o						30
		4: total	++	++	o	o	+++		+++		o		++	31 - 38
		m	+	+	+	(+)	b		b		+		o	
		s	-	-	-	(-)	++		++		-		+	
D4	mitotic segregation	1										+	+	39
	crossing-overs	4: total	+++								+		o	40 - 42
	non-disjunctions	4: total	+								++		+	40 - 42
		m	+								+			
		s	-								+			
D7	crossing-overs	4: total	+++		(+)	o			+	o	+	+	+	44 - 51
		m	+		(+)	o			(+)	+				
		s	o		o	+			(+)	-				
	non-disjunctions	4: total	+		o	o			+	(+)	++	++	++	44 - 51
		m	+		+	o			+	+	+	+	o	
		s	-		-	f			f	-	-	(+)	+	
D3	recessive lethals		+	+										52

Legend:

- m effect on the spontaneous mutant frequency caused by mutagenicity
- s effect on the spontaneous mutant frequency caused by selective advantage
- b m could not be measured since the selective advantage was too high
- f very great differences between the repetitions

+++	very large increase, significant	Note: significance is not calculated for m and s
++	large increase, significant	
+	increase, significant	
(+)	small increase, not significant	
o	no effect	
(-)	small decrease (only for s)	
-	decrease (only for s)	

carcinogenic (Bruce & Heddle, 1979; McCann et al., 1975). In Aspergillus, the induction of point mutations, crossing-overs, gene conversions and non-disjunctions have been reported (Gualandi et al., 1979; de Bertoldi et al., 1980).

In our study MMS has, as expected, been active in all test systems: induction of point mutations, deletions and / or crossing-overs from strain 007, crossing-overs in the strains D4 and D7, and recessive lethals. Also an increase in non-disjunction products of the strains D4 and D7 has been found. It is, however, possible that an unknown number of colonies scored as non-disjunction products actually arose after a double cross-over which can lead to colonies with the same phenotype.

BED, too, has been found to cause several types of mutations, like point mutations in Salmonella, mitotic gene conversions in Saccharomyces and recessive lethals in Drosophila. BED is reported to be carcinogenic (IARC monographs, vol. 11, 1976). With Aspergillus as a test organism, point mutations and crossing-overs have been reported (Alderson & Hartly, 1969; Morpurgo, 1963).

In the present study, BED was found to induce point mutations, deletions and / or crossing-overs in strain 007 and recessive lethals.

Nitrite (NO2) is known to react with amino groups resulting in, among other things, a deamination of the primary amines. Deaminated cytosine pairs with uracil and deaminated adenine with xanthine, both resulting in base transitions. Deaminated guanine cannot pair at all, and repair mechanisms, which may introduce errors, must restore a correct DNA strand (Freese, 1971).

Especially in the undissociated state at low pH, NO2 is known to be a strong mutagen for several bacteria, for Neurospora, Aspergillus and Saccharomyces. It also induces crossing-overs and gene conversions in yeast, and chromosome breaks in lillyflower (Zimmermann, 1977). It is, however, not (directly) carcinogenic (McCann et al., 1975).

In the present study NO2, always tested at pH 4.5, gave different results depending on whether incubation method 2 or 4 was used. Both the number of point mutations (strain 110) and deletions and / or crossing-overs (strain 007) have been found to be enhanced with method 2. With method 4, only on strain 007 a weak effect on the recombination frequency was found. In all cases a difference between

the fitness of the mutants / recombinants and the test strains has been observed. In testing strain D7 (non-disjunctions) a real recombinogenic activity has possibly been obscured by a selective disadvantage of the recombinants, but this disadvantage is so extreme that the positive effect (on the recombinogenicity) must be interpreted with caution (see discussion in 8.1.4).

Azide (N_3) is known to react with cells in many ways. Many enzymes are inhibited, among which those of oxydative phosphorylation and DNA synthesis (Kleinhofs et al., 1978). The inability to use acetate as a carbon source after N_3 treatment (table 27) is probably caused by inhibition of specific enzymes needed to metabolize this substrate. The mutagenic activity, however, is not understood. Generally a low pH is needed, thus the undissociated HN_3 may be involved. An intermediate of the azide metabolism (azido alanine) is known to occur which has the same mutagenic properties as N_3 itself and which might be the real mutagenic compound (Owais et al., 1983).

N_3 is strongly mutagenic to some organisms (bacteria, Saccharomyces and barley) but not mutagenic to others (Neurospora, Arabidopsis, Drosophila and mammals) and is not carcinogenic (Kleinhofs et al., 1978). This difference might be caused by a different azido alanine catabolism (Rosichan et al., 1983).

In the present study a strong positive effect was observed with incubation method 2, both with strain 110 and 007, at pH 4.5. However, when tested with method 4 in no case a higher number of mutants or recombinants was observed. A selection effect was always found but the only effect on the mutagenicity factor has been with strain 007.

Since in some of the tests a positive effect is observed, both NO_2 and N_3 must be regarded as mutagenic / recombinogenic. The difference between the results obtained with both incubation methods will be discussed in chapter 8.4.

Two acridines have been tested in this study: 9-aminoacridine (9AA) and acriflavine (ACR). Both intercalating agents have been reported to be mutagenic to several organisms, but not to others, as reviewed by Nasim and Brychy (1979). Some of the effects of 9AA are the induction of frameshifts in E. coli, increased sex-linked and recessive lethal

damage in Drosophila and the induction of chromosome mutations in barley. ACR induces mutations in E. coli and Salmonella, gene conversions in yeast, sex-linked lethals in Drosophila, chromosome breaks in Allium, sister chromatid exchange in chinese hamsters and dominant lethals in mice. The carcinogenicity data are inconclusive.

In our study 9AA and ACR show different results. 9AA has not been found to be active with the strains 110 and 007 when method 2 and 3 are used (mostly DMSO has been used as a solvent). Surprisingly, with method 4, the effect on strain 110 was found to depend on whether DMSO is used as a solvent or not. With DMSO no effect is seen, but when 9AA is directly solved in the warm, melted agar, a clear increase of the mutant frequency can be observed. Burnett et al. (1982), testing the hair dye p-phenylenediamine by the Ames test, also reported a solvent effect of DMSO. But here the effect pointed in the opposite direction, since in their study a mutagenic action was only shown when the dye remained for several hours in DMSO before use.

Tested with strain 007 (without solvent), an extreme selective advantage of the segregants has been observed and consequently a possible recombinogenic effect will not have been observed. The results reported here are in sharp contrast to those of Bignami et al. (1982), who found a strong mutagenic effect of 9AA, solved in DMSO, when applied to Aspergillus and using an incubation method comparable to our method 2.

In contrast, ACR (solved in H_2O) induced weakly, but significantly, point mutations at comparable concentrations, and at a low survival rate. Also an effect on strain 007 was observed. However, with method 4, no other effect was found on strain 110 than a selective advantage of the mutants. On strain 007 (tested with method 4) ACR acted just like 9AA, giving an extreme advantage of the segregants.

Both para-fluorophenylalanine (FPA) and chloralhydrate (CH) are well-known inducers of non-disjunctions and are used to force diploid Aspergillus strains to haploidize (Lhoas, 1961; Singh & Sinha, 1976). But also other weak effects have been reported. FPA induces gene conversions and forward mutations in yeast (Davies & Parry, 1978) and forward and reverse mutations in Ustilago maydis (Lewis & Tarrant, 1971). CH is reported to be mutagenic to Salmonella, Aspergillus and Streptomyces (Bignami et al., 1980).

In our study FPA has been tested with D7 / method 4 and, surprisingly, the numbers of non-disjunction products were not found to be enhanced. It turned out that the real recombinogenic effect of FPA is obscured by a selective disadvantage of the non-disjunction products. To a lesser extent this was also found for the crossing-over products. This is curious since in higher concentrations FPA is regularly used in selecting haploids. In our study higher concentrations FPA could not be tested since the conidiation was so much affected that too few conidia were recovered.

The ability of CH to induce non-disjunctions has clearly been demonstrated on strain D4 and D7 (method 4). The numbers of non-disjunction products were always enhanced. Some increase of the numbers of crossing-over products was also observed, but no attempt was made to distinguish recombinogenic from selective processes. A weak mutagenic action on strain 007 was obscured by selective disadvantage of the segregants. Our results confirm that the main action of CH is on the non-disjunction frequency, but other processes are also induced.

The fungicide carbendazim is known to induce non-disjunctions in Aspergillus, mitotic abnormalities in bone marrow of rats and point mutations in excision repair deficient cells of Aspergillus (Kappas, 1981).

In our study no effect on strain 110 has been observed (method 4). Mitotic segregation of strain D4 (method 1) is enhanced, probably by an effect on the number of non-disjunctions since, when tested with method 4, this was found to be the most important effect. However, some effect on the crossing-over frequency has been observed, too.

Vinclozolin, the other fungicide tested, is reported to have some genetic activity. It is weakly mutagenic in Salmonella (Chiesara et al., 1982) and induces non-disjunctions in Aspergillus (Vallini et al., 1983).

In the present study no effect on strain 110 has been seen (method 4), but higher numbers of yellow recombinants from strain 007 (method 4), sectors from strain D4 (method 1), non-disjunctions with strain D4 and D7 (method 4) and crossing-overs with strain D7 (method 4) were observed. In all cases where it could be calculated the only effect was a selective advantage of the recombinants. Thereby the non

-disjunction inducing ability of vinclozolin is not confirmed. Vallini et al. (1983) tested vinclozolin using an incubation method equal to our method 3, where the conidia were incubated for 3 hours in the presence of vincozolin. It is not likely that during such a short period of time, a selective advantage can have such a great effect on the recombinant frequency.

8.4 Comparison of the test results of the different incubation methods

Comparing the effect on the strains 007 and 110 it is noticed that, although the extent to which the spontaneous frequency is enhanced is very different (110 is more enhanced than 007), the effects of the 6 chemicals tested on both strains with method 2 are quantitatively similar. So no evidence is found that strain 007 can give information which is not obtained by using strain 110. Only with method 4 some difference (NO2) is found.

Using method 4, some chemicals (NO2, N3 and ACR)- which have known mutagenic effects and indeed were mutagenic when method 2 was used - did not give a higher proportion of mutants in strain 110, although method 4 might be expected to be in general more sensitive than method 2. There are a number of fundamental differences between the methods 2 and 4:

- the mutagen concentration is usually lower with method 4.
- while the pH stays at 4.5 or 6.8 with method 2, the pH rises during mycelial growth to 7.6 with method 4.
- the incubation time with method 4 is much longer than with method 2 and during this incubation the concentration of a reactive compound will decrease and metabolites will arise.
- with method 4, the fungus is tested during all mitotic cell stages, while with method 2 only dormant conidia are tested.
- with method 2, the suspension is incubated under continuous white light, while the plates of method 4 are incubated in the dark.

The latter point may explain the ACR mutagenicity data, since this compound is known to be more reactive in light (Ball & Roper, 1966). The related chemical 9AA showed the opposite effect (not active with method 2 but active with method 4), but here the differential effect might be caused by interaction with DMSO. Both NO2 and N3 need a low pH for their mutagenic action but it is not likely that the pH-change

causes the differential action with the two methods. The initial mutations (at pH 4.5) will contribute much more to the finally observed mutant frequency than mutations which arise late in mycelial growth. Other differences must have played a role as well.

With method 4 the control is imperative at least when testing the strains 007, D4 and D7, since here there are considerable differences between the genotype of the test strains and their recombinants, possibly resulting in a difference in fitness. For example, the effect of vinclozolin, as far as tested, was found to be caused by a selective advantage, whereas chloralhydrate was really recombinogenic.

Our investigations confirm that Aspergillus nidulans can readily be used as a test organism for mutagenicity testing. It should be kept in mind that the systems used in the present study can be made more sensitive in several ways, analogous to the Ames test. It is possible to add mammaliam enzymes for metabolic activation as done by de Bertoldi et al. (1980), and alleles responsible for a deficient excision repair can also be introduced to the test strain (Kappas & Bridges, 1981). In this way the systems can be improved.

8.5 Summary of main conclusions

- Comparing the incubation methods, it can be concluded that the following two incubation methods are the best:

1 The liquid suspension test (method 2) can readily be used for measuring the mutagenicity and recombinogenicity of agents acting directly on dormant conidia.

2 The media mediated assay (method 4) is most suitable when germinating conidia and growing mycelium are to be tested. However, an additional control experiment has to be done in order to distinguish between mutagenicity and selection processes.

The plate incorporation assay (method 1) may be used for rapid pre-screening tests.

The liquid test (method 3) was not satisfactory in testing germinating conidia.

- The methionine deficient strain 110 is very suitable for measuring the point mutation frequency.

- The construction of a duplication strain heterozygous for the sorA allele (strain 007) reduces the labour of measuring the instability of duplication strains. In the present study, however, no evidence was found that other kinds of mutagens may be discovered than with the system used for measuring the point mutation frequency. Of course, more and other kinds of chemicals have to be tested to generalize the latter conclusion.

- The crossing-over and non-disjunction frequencies can be estimated by observing mitotic segregation, but when the two processes are to be distinguished, selective systems must be preferred. Selection of pimaricin resistant recombinants proved to be the most suitable system, in combination with the media mediated assay.

- The recognition of recessive lethal mutations is too laborious for routine use.

- For routine use, we recommend as the most important test systems:

Strain 110 (point mutations) - liquid suspension test
Strain D7 (mitotic crossing -overs and non-disjunctions) - media mediated assay

When additional information is required these tests can be supplemented with:

Strain 110 (point mutations) - media mediated assay
Strain 007 (deletions and / or crossing-overs) - liquid suspension test or media mediated assay

For certain purposes the frequency of recessive lethals (strain D3) can be measured, or all abnormal colonies (strain D4) can be isolated.

Recommended protocols for these selected test systems are shown in the appendix.

SUMMARY

The suitability of Aspergillus nidulans as a test organism for mutagenicity testing has been investigated. Several test systems have been tried. Those systems were preferred in which the mutants or recombinants could be selected without the need of adding a compound to the medium which itself is genetically active.

Four incubation methods have been compared. The plate incorporation assay (method 1) is the easiest method for testing chemical compounds, but it is often difficult to obtain reliable quantitative data. Moreover, complications may arise when the compound is toxic or causes a selective advantage of the mutants. In the liquid suspension test (method 2) the conditions can be controlled more accurately, so this method is more suitable for obtaining reliable quantitative data. For testing germinating conidia, the suspension test (method 3) turned out to be less suitable, since some conidia germinated directly from the start causing - in spite of the presence of 0.2% agar - big clumps of conidia at the end of the incubation period. Germinating conidia (and growing mycelium) can better be tested with the media mediated assay (method 4). However, an extra control experiment must be performed in order to discriminate between a real mutagenic activity and a selective advantage.

Strain 110, which carries the methG1 allele, proved to be very suitable for measuring the point mutation frequency when methionine independent suppressor mutants are scored. The instability of a duplication strain can be also used as an indication for the genetic activity of a chemical compound. The construction of a duplication strain heterozygous for the recessive sorA2 allele (strain 007) reduced the number of plates needed for the scoring of segregants. The exact process by which the sectors arise is not known. Some crossing-over products are scored as deletion products when no special attention is paid to the morphology of the segregants.

For determining the crossing-over and non-disjunction frequencies scoring of mitotic segregation is the easiest way, at least when it is not intended to distinguish the inducing processes. If such a distinction is required, it is easier to select the recombinants. Strain D4

can be used to select recombinants homozygous for the suAladE20 allele, and the crossing-over and non-disjunction products can be directly distinguished by their conidial colour. In practice, complications arose since the conidial colour was faint and the leaky growth of the test strain led to a concentration effect. Selection of sorbose resistant recombinants was even less suitable, and a combinated selection of sorbose resistant and adenine prototroph recombinants proved unfeasible. Selection of pimaricin resistant recombinants of strain D7 was much easier and therefore preferably used in this study. However, since pimaricin itself is known to induce non-disjunctions this system has only been used in combination with the media mediated assay to minimize the risk of complications.

The system for measuring the frequency of recessive lethals developed by Morpurgo et al. (1978) has also been investigated, but the background in the control was relatively high. This was most likely caused by incomplete haploidization on FPA medium. Changing the medium did hardly improve the results. This implies that one can only measure larger effects on the frequency of recessive lethals.

Several chemicals, mostly with known mutagenic properties have been tested by means of the proposed test systems. These confirmed the suitability of the test systems.

Thus, in addition to other mutagenicity tests like the Ames test with bacteria, the fungus Aspergillus nidulans offers good opportunities to asses the genetic activity of chemical compounds on eucaryotic organisms.

SAMENVATTING

De geschiktheid van de schimmel Aspergillus nidulans om te dienen als testorganisme voor het doorvoeren van mutageniteitstests werd onderzocht. Verscheidene mogelijkheden werden vergeleken. Hierbij werd de voorkeur gegeven aan systemen waarmee de gezochte mutanten of rekombinanten geselekteerd konden worden, zonder dat er een stof aan het medium moet worden toegevoegd die zelf mutageen is.

Er werden 4 inkubatiemethoden vergeleken. De eerste methode is in principe de eenvoudigste. Hierbij wordt de te onderzoeken stof direkt toegevoegd aan de selektieplaat. Het is echter vaak moeilijk om betrouwbare kwantitatieve gegevens te verkrijgen. Er kunnen komplikaties optreden wanneer de onderzochte stof toxisch is, zeker als er verschillen zijn in inwerking op de teststam en op de mutanten of rekombinanten. Bij gebruik van de test in vloeibaar medium (methode 2) kunnen de omstandigheden beter gekontroleerd worden en hierdoor is deze methode beter geschikt om kwantitatieve gegevens te verkrijgen. Voor het testen van kiemende sporen bleek de test in vloeibaar medium, in aanwezigheid van 0.2% agar (methode 3), minder geschikt, want aan het eind van de inkubatieperiode bleken de sporen toch gedeeltelijk samengeklonterd te zijn. De test in kompleet medium (methode 4) is beter geschikt voor het testen van kiemende sporen (en groeiend mycelium). Er is echter een extra kontrole nodig om echte mutagene eigenschappen te kunnen onderscheiden van selektie invloeden.

Het scoren van methionine onafhankelijke suppressor mutanten van stam 110 bleek een goede mogelijkheid te zijn voor het meten van de puntmutatie frekwentie. De instabiliteit van een duplikatiestam kan eveneens gebruikt worden voor het vaststellen of een te onderzoeken stof inwerkt op het genetische materiaal. Door het inbouwen van het sorA2 allel in de (I→II) duplikatie (stam 007) is het gemakkelijker geworden om het aantal segreganten te tellen. Het is echter niet precies bekend waardoor deze instabiliteit wordt veroorzaakt. Ons onderzoek toont aan dat ook bepaalde crossing-over produkten gescoord worden als deletie produkten, als er geen speciale aandacht geschonken wordt aan de morphologie van de segreganten.

Als het veroorzakende mechanisme niet bekend hoeft te worden is het

voor het meten van de crossing-over en non-disjunktie frekwentie het eenvoudigst om het ontstaan van sektoren met recessieve kleuren te scoren. Als het mechanisme wel bekend moet worden is het eenvoudiger om op rekombinanten te selekteren. Het suAladE20 allel, aanwezig in stam D4, kan hiervoor gebruikt worden, omdat de crossing-over en non-disjunktie produkten direkt onderscheiden kunnen worden vanwege hun kleur. In de praktijk treden er echter komplikaties op, omdat de kleur nogal bleek is en omdat de teststam toch nog wat groeit op medium zonder adenine. Hierdoor onstaat een koncentratie-effekt. Het selekteren op sorbose resistentie bleek nog minder geschikt, terwijl een gekombineerde selektie op sorbose resistente en adenine prototrophe rekombinanten niet mogelijk bleek te zijn. Het selekteren van pimaricine resistente rekombinanten van stam D7 is veel gemakkelijker en is daarom bij voorkeur gebruikt in dit onderzoek. Omdat pimaricine zelf non-disjunkties kan veroorzaken is dit systeem alleen gebruikt in kombinatie met inkubatiemethode 4.

De methode van Morpurgo et al. (1978) om recessief lethalen aan te tonen is ook toegepast. Er werd echter altijd een zwak positief effekt gevonden in de kontrole, wat er op duidt dat de haploïdizatie d.m.v. FPA niet volledig was. Veranderingen in het medium gaven geen verbetering. Dit betekent dat met dit systeem alleen grote effekten gemeten kunnen worden.

Verscheidene chemikaliën, meestal met bekende mutagene eigenschappen, werden getest met de voorgestelde testsystemen. Ze bevestigen de geschiktheid van deze testsystemen.

Onze konklusie is dat de schimmel Aspergillus nidulans heel goed gebruikt kan worden om, in aanvulling op andere mutageniteitstests zoals de Amestest, gegevens te verkrijgen over de genetische aktiviteit van chemikaliën op eukaryote organismen.

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APPENDIX

Recommended test records for the most important systems

A Liquid suspension test with strain 110:

1 Preparation of a conidial suspension.

2 Incubation of a suspension of ca. 5×10^6 conidia / ml in 25 ml buffer + the chemical to be tested in a 100 ml flask. This suspension is shaken for 1 or 2 hours at 37 °C.

3 At termination of the incubation, at first a probe is plated in an appropriate dilution on CM + glu + des and then the incubation mixture is filtrated and the remaining conidia are washed.

4 The conidia are resuspended and the concentration of the resulting suspension is estimated by using a Coulter Counter.

5 Then this suspension is plated:
- on MM + glu + bio: two different concentrations (usually 2×10^6 and 4×10^5 con / plate) are plated using 5 plates at each concentration.
- on CM + glu + des: at least 10 plates are inoculated with ca. 100 colonies per plate.

6 After 2 days at 37 °C the colonies on the CM plates are counted.

7 After 4 days at 37 °C the revertant colonies on the MM plates are counted if there are no more than 25 colonies on one plate.

B Media mediated assay with strain D7:

1 Preparation of a conidial suspension of D7 and also of the yellow and green recombinant types.

2 The conidial concentrations are estimated using a Coulter Counter. The suspension of conidia of D7 is diluted to 5×10^7 con / ml. Also a mixt suspension is made: 5×10^7 conidia of D7 and 1.25×10^5 conidia of both recombinant types per ml.

3 Plates CM + glu are poured with and without the chemical to be tested. After these plates have become solid, 0.1 ml of the suspensions mentioned before are inoculated. The plates are incubated at 37 °C.

4 The conidiation is scored after 3 (or 4) days and a conidial suspension is made from each plate. The concentration is estimated using a Coulter Counter.

5 Then these suspensions are plated:
- on MEA + glu + paba, pyro, pro + PIM: two concentrations are used; for the normal suspensions: 2×10^5 and 2×10^6 con. / plate, and for the suspensions artificially enriched with recombinants: 5×10^3 and 5×10^4 con. / plate. Three or more plates are used for each concentration.
- on CM + glu + des: at least 10 plates are inoculated with ca. 100 colonies per plate.

6 After 2 days at 37 °C the colonies on the CM plates are counted.

7 After 4 days at 37 °C the green and yellow colonies on the MEA plates are counted separately.

C Media mediated assay with strain 110:

1 Preparation of a conidial suspension of 110 and of its mutant types A, B and C.

2 The conidial concentrations are estimated using a Coulter Counter. The suspension of conidia of 110 is diluted to 5×10^7 con. / ml. Also a mixt suspension is made: 5×10^7 conidia of 110 and 0.75, 2.5 and 0.38×10^3 conidia of the mutant types A, B and C respectively per ml.

3 Plates CM + glu are poured with and without the chemical to be tested. After these plates have become solid, 0.1 ml of the suspensions mentioned before are inoculated. The plates are incubated at 37 °C.

4 The conidiation is scored after 3 (or 4) days and a conidial suspension is made from each plate. The concentration is estimated using a Coulter Counter.

5 - 7 These steps are identical to those of the liquid suspension test with strain 110 (A).

D Liquid suspension test with strain 007:

1 - 4 These steps are identical to those of the liquid suspension test with strain 110 (A).

5 The suspension is plated:
- on MM + ace + paba, pro + SOR: two different concentrations (usually 1×10^5 and 2×10^4 con. / plate) are plated using 5 plates at each concentration.
- on CM + glu + des: at least 10 plates are inoculated with ca. 100 colonies per plate.

6 The CM plates are incubated at 37 °C and after 2 days the colonies are counted.

7 The MM plates are firstly incubated for 1 day at 37 °C, then for 3 days at 25 °C and at last 1 day at 37 °C. All visible yellow colonies are counted.

E Media mediated assay with strain 007:

1 Preparation of a conidial suspension of 007 and of the yellow recombinant types.

2 The conidial concentrations are estimated using a Coulter Counter. The suspension of conidia of 007 is diluted to 5×10^7 con. / ml. Also a mixt suspension is made: 5×10^7 conidia of 007 and 1.25×10^4 recombinant conidia.

3 - 4 These steps are identical to those of the media mediated assay with strain D7 (B).

5 - 7 These steps are identical to those of the liquid suspension test with strain 007 (D).

CURRICULUM VITAE

Johan Gerrit Boschloo, geboren op 21 mei 1954 te Teuge, behaalde in 1972 het diploma HBS-B aan het Christelijk Lyceum te Apeldoorn, waarna hij aan de Landbouwhogeschool ging studeren. In 1979 werd het doctoraal examen Moleculaire Wetenschappen afgelegd, met als hoofdvakken de erfelijkheidsleer en de virologie en als bijvak de biochemie. Van 1 april 1980 tot 31 maart 1984 was hij als wetenschappelijk medewerker verbonden aan het "Institut für Pflanzenpathologie und Pflanzenschutz" van de universiteit te Göttingen en verwerkte daarna de in deze periode verkregen gegevens tot dit proefschrift.