MEIOTIC ANEUPLOIDY AND ITS INDUCTION IN
SORDARIA BREVICOLLIS

by

ALEXANDRA M. FULTON

Ph.D
University of Edinburgh
1983
This thesis was composed by myself and describes my own work, except where otherwise stated either in the acknowledgements or in the text.
ACKNOWLEDGEMENTS

I am indebted to Dr. D.J. Bond for his help and encouragement throughout the course of this project, both by discussion and practical assistance. The aneuploid testing experiments described in chapters 5 and 6 required large numbers of slides to be made up and I am grateful to Dr. Bond, who assembled some of these slides and helped in dissecting the aneuploid-containing asci from the 5000rad cross in chapter 6. I would also like to thank Lynn McMillan for assistance at the beginning of this project. The linkage group IV strains used for aneuploid testing were kindly supplied by Dr. H.L. K. Whitehouse. I am grateful to Dr. T. F. MacKay, Dr. W. G. Hill and Prof. D. S. Falconer for advice on statistical tests. The photographs were developed and printed by Alan McEwan and Jim Forrest of the photography department. The X-ray source was calibrated by Dr. G. G. Selman. Thanks are also due to Jackie Bogie and Ann Brown for all their help through their postal typing service and to Dr. A. J. Kingsman and Dr. S. Kingsman for their understanding while I completed this thesis. I thank Prof. J. R. S. Finchman for the provision of laboratory facilities and the Department of Education for Northern Ireland for financial support.
ABSTRACT

Meiotic aneuploidy and its effects on man are considered, and the need for a system to detect meiotic aneuploidy is discussed. Various errors which can give rise to aneuploidy are presented and aneuploidy detection systems available in mammals, Drosophila and fungi are reviewed. Lower eukaryotic systems are desirable for reasons of speed and economy. Two aneuploid detection systems in Sordaria brevicollis are investigated. These are based on complementation of alleles of spore colour mutant loci, $\text{buff-1}$, and $\text{grey-6}$, on linkage groups II and IV respectively. These systems detect a range of meiotic errors; first division non-disjunction and non-conjunction, second division non-disjunction and premature centromere division.

The spontaneous frequencies of aneuploidy for each system were studied. The systems were shown to be susceptible to induction by p-fluorophenylalanine, which affected both the first and the second meiotic division, but caused greater increases in second division errors.

The sensitivity of the linkage group II system to induction by spindle inhibitors was studied. Spindle poisons, colchicine and vinblastine, cause severe effects at low concentrations, in mammals, but did not induce aneuploidy in the Sordaria system even at relatively high concentrations. Surprisingly, methyl-2-benzimidazole carbamate and
benomyl, which are potent fungicides acting on the fungal spindle, did not induce aneuploidy above levels obtained with the dimethylsulfoxide solvent control. It is possible that meiotic divisions have not been exposed to these compounds.

X-rays are well known aneuploid inducers in *Drosophila*, where the main increase is thought to be through chromosome breakage giving rise to tertiary aneuploidy. In *Sordaria* X-rays had little effect on primary aneuploidy but did increase tertiary aneuploidy. Five strains were isolated which exhibited increased aneuploid frequency. Preliminary analysis suggested two of these strains carried a reciprocal translocation and one an insertional translocation. A fourth possible rearrangement was not analysed due to lack of material. The fifth strain is potentially very interesting. This exhibited a high frequency of premature centromere division in heterozygous crosses. Linkage analysis did not indicate involvement of any other linkage group. This strain may carry a very small chromosome rearrangement or be a meiotic mutant.

Attempts to increase the efficiency of the *Sordaria* aneuploid detection system by developing a random spore plating method are described. A method was not fully developed but some of the problems involved are discussed.
# TABLE OF CONTENTS

## CHAPTER 1 INTRODUCTION
- The consequences of aneuploidy ........................................... 1
- The frequency of aneuploidy in man and other mammals ............. 4
- Concern about environmental agents inducing aneuploidy ........... 14
- Meiotic errors resulting in aneuploidy .................................. 18
- Testing systems ....................................................................... 33
- Aims of this project .................................................................. 66

## CHAPTER 2 MATERIALS AND METHODS
- Strains used .............................................................................. 68
- Crossing techniques ................................................................. 71
- Harvesting crosses and storing material .................................... 72
- Scoring crosses for aneuploidy .................................................. 73
- Mating type tests ....................................................................... 74
- Random spore isolation and ascus dissection ............................. 75
- Auxotrophy tests ........................................................................ 75
- Mutant hunts .............................................................................. 76
- Statistical procedures ............................................................... 79

## CHAPTER 3 SPONTANEOUS ANEUPLOIDY INVOLVING LINKAGE GROUP II OR LINKAGE IV OF SORDARIA BREVICOLLIS
- The buff aneuploid detection system .......................................... 81
- Development of the grey-6 aneuploid detection system ............... 107
- Comparison of the relative proportion of meiotic errors occurring in Neurospora and Sordaria ....................................................... 122
- Investigating non-conjunction and non-disjunction at the first meiotic division ................................................................. 123
- Summary ...................................................................................... 127
CHAPTER 4  ANEUPLOID INDUCTION BY PARAFLUOROPHENYLALANINE  130

Introduction  130
Results  131
Does the treatment affect the overall frequency  132
of aneuploidy?
Is there a dose related effect?  134
Is the distribution of ascus types altered?  138
Recombination and pFPA induction  149
Time of application and induction by pFPA  151
The action of pFPA at meiosis  152
Summary  159

CHAPTER 5  SPINDLE INHIBITORS AND MEIOTIC ANEUPLOIDY IN
SORDARIA BREVICOLLIS  161

Introduction  161
Materials and methods  163
The effects of spindle inhibitors on aneuploidy  164
Colchicine  164
Vinblastine  168
Benomyl and methyl-2-benzimidazole carbamate  172
Tubulin extraction  178
The effect of DMSO on aneuploidy in Sordaria  180
Discussion  187

CHAPTER 6  X-RAY INDUCTION OF ANEUPLOIDY IN SORDARIA
BREVICOLLIS  192

Introduction  192
Irradiation  197
Results  198
The effect of X-rays on aneuploid frequency  198
in Sordaria
The effect of X-rays on recombination at the
grey-6 locus  202
Analysis of the multiple aneuploid rosettes(MAR)  203
Discussion  226
CHAPTER 1: INTRODUCTION

The term aneuploid was first used by Täckholm (1922) to describe the chromosome numbers of polyploid rose hybrids which were not simple multiples of the basic chromosome number. It is now a widely used term and an aneuploid organism is defined as having a chromosome number unequal to the haploid number, n, or any simple multiple thereof. Aneuploidy arises through errors in chromosome distribution at either mitosis or meiosis. In diploids the commonest aneuploid chromosome numbers are $2n + 1$ and $2n - 1$ and these are described as trisomic and monosomic for the chromosomes involved. The equivalent results for haploids are disomy $(n + 1)$ and nullisomy $(n - 1)$.

Another term frequently used in connection with aneuploidy is non-disjunction. This was first used by Bridges (1913) and describes one mechanism by which aneuploidy can arise, that is through non-separation of homologues or of sister chromatids. Aneuploidy can arise through various mitotic or meiotic errors and the term non-disjunction has been extended to include any error giving rise to aneuploidy. The use of the term non-disjunction in this wider sense is very common in the literature, as no more general term is available.

1. The consequences of aneuploidy

The consequences of the aneuploid condition to an organism depend both on the organism and on which chromosome or chromosomes are abnormally represented. A few examples illustrate this point. In yeast stocks can be
maintained of singly and multiply disomic strains but disomy is more readily tolerated for some chromosomes than for others (Parry and Cox, 1970). In diploid strains the stability of monosomics depends on the chromosome involved (Bruenn and Mortimer, 1970). In _Neurospora crassa_ and _Sordaria brevicollis_ aneuploids are unstable and break down to give heterokaryotic cultures (Pittenger, 1954; Bond, unpublished). In _Drosophila melanogaster_ it is possible to maintain trisomic stocks involving the X chromosome or fourth chromosome, (Lindsley and Grell, 1967) but aneuploidy for the second or third chromosome is lethal. In mice XXY, XO and XYY offspring have been found (Cattanach, 1961; Cattanach, 1962 and Cattanach and Pollard, 1969), but there are few instances of liveborn autosomal trisomics. Trisomy 19 has been shown to be compatible with survival to birth but not beyond this point (White _et al._, 1972).

In man aneuploidy has very severe effects ranging from spontaneous abortion of pregnancy to various recognised syndromes among liveborns, such as Down's syndrome (trisomy 21), Edward's syndrome (trisomy 18), Patau's syndrome (trisomy 13) and various sex chromosome aneuploidies (XO, XYY, XXX and XXY). On rare occasions, trisomies 7, 8, 9 and 22 have been known to survive to term but have no syndrome names attached to them (Sankaranarayanan, 1979).

Recognition of all these chromosome anomalies is possible because of the well characterised human karyotype, with chromosome pairs being identifiable by their distinct banding patterns with quinacrine fluorescence (Q-banding)
and Giesma staining (G-banding). A standard nomenclature has been agreed and is continuously updated (I.S.C.N. 1981). All of the autosomal syndromes show physical abnormalities and severe mental retardation and result in reduced life expectancy. Sex chromosomal aneuploidy has less severe effects resulting in varying degrees of mental retardation and causing sterility or reduced fertility.

Hook (1982) considers the contribution of chromosome anomalies to human morbidity and mortality. Among live-borns there are about 0.8% congenital heart defects of which approximately 10% are associated with numerical chromosome abnormalities. Surveys among patients in institutes for the mentally retarded (I.Q. <50) in Australia, Japan, the United States of America and the United Kingdom reported 8-15% chromosome abnormalities. Earlier studies gave much higher incidences of Down's syndrome alone, and Hook suggests that at least part of the reason for the decrease is the increasing tendency to keep Down's syndrome children at home. The incidence of aneuploidy among those with moderate retardation, defined as having an I.Q. between 50 and 69, is not well known, but Hook gives a crude estimate of 7-15%.

Chromosome abnormalities are also associated with infertility. Hook reports studies on peripheral blood giving estimates of 2.1 - 3.5% of infertile or subfertile males with chromosome abnormalities.

The severe effects of numerical chromosome anomalies place a high load on society, both in human distress and
in the financial requirements for care of the affected individuals. In order to gain some idea of the frequency of meiotic errors in man several surveys have been carried out. These surveys have ranged from studies to determine the frequency at conception and in early pregnancy to studies on liveborns. As far as the load on society is concerned the most important results are those concerning liveborns and to a lesser extent stillborns and recognised spontaneous abortions. The frequency of events at conception is really only of academic interest, as embryos which are aborted before pregnancy has been recognised do not cause any distress or expenditure. However, in order to fully appreciate the effects of aneuploidy in man, a brief outline of studies at all stages will be given.

2. The frequency of aneuploidy in man and other mammals
   a. The frequency of aneuploidy in man

It has proved difficult to estimate the frequency of aneuploidy in man. Estimates of the frequency of aneuploidy at conception vary from 8% by Alberman and Creasy (1977) to 50% by Boué et al. (1975). This variation comes about because there are some complicating factors which result in there being large errors associated with the estimation procedure.

One of the most striking things about aneuploidy in man is the pronounced maternal age effect, that is an increased frequency of aneuploid offspring born to older women. This was first reported for trisomy 21 by Penrose (1933) and has since been shown to hold for all autosomal
trisomic conditions (Hassold et al., 1980). Trisomy 16 is different from trisomy of the other autosomes. It occurs at a much higher frequency amongst spontaneous abortions than other trisomics, and has a very much smaller maternal age effect. There is also an age effect for sex chromosome trisomics but not for XO conceptuses (Court-Brown et al., 1969). The maternal age effect is found among liveborns (Sankaranarayanan, 1979) and among spontaneous abortions (Hassold et al., 1980). Estimates of the frequency of aneuploidy in man will, therefore, vary according to the mean maternal age of the sample.

Another factor affecting estimates is that most aneuploid conceptuses abort spontaneously before full term, so that those liveborn individuals which are aneuploid represent only a small fraction of those conceived. The earlier in pregnancy that mothers are examined the higher the frequency of aneuploid zygotes. In analysis of spontaneous abortions the most important factor in determining the observed frequency of aneuploidy is the gestational age of the conceptus. As estimates of the frequency of aneuploidy at conception make use of information on the frequencies at other stages of pregnancy the studies on aneuploidy will be reviewed for liveborns, spontaneous abortions and then conceptions.

Aneuploidy among stillborns, neonatal deaths and liveborns

The frequency of chromosome anomalies among stillborns and neonatal deaths was found to be 5-6% (Machin and Crolla, 1974) with 56% of these being autosomal trisomics and 21%
sex chromosome anomalies. A summary of six major surveys of newborns gave data on 56,900 children (Hook and Hamerton, 1977). Frequencies ranged from 0.5% to 0.8% with the overall frequency being 0.62%. Breakdown of this data showed that 0.14% of newborns had autosomal trisomies, 0.22% sex chromosomal anomalies and 0.26% structural chromosome anomalies. Sex and autosomal trisomies made up 48.7% of the chromosome anomalies.

Aneuploidy among spontaneous abortions

Boué et al. (1975) studied spontaneous abortions of embryos less than twelve weeks old. Of 1,498 abortions 60% showed chromosome anomalies of which 67% were simple monosomies or trisomies. This was a very comprehensive study which took into account patients who aborted at home and excluded, as far as was possible, induced abortions. The disadvantage of the study was that it only included embryos up to twelve weeks gestational age. This would cause an excess of growth retarded conceptuses to be included in the study and these have a high frequency of chromosome anomalies (Carr and Gedeon, 1977).

Creasy et al. (1976) obtained an estimate of 30.5% chromosome anomalies among spontaneous abortions of foetuses up to 27 weeks after conception. Of these 49.8% were trisomic and 23.7% XO. Only hospitalised patients were sampled, and this probably biased against early abortions, so lowering the estimate. The actual figure probably lies between 30.5% and 60%.

Carr and Gedem (1977) summarised eight studies of spontaneous abortions and found that, of the chromosomally
abnormal spontaneous abortions 52% were trisomic, 18% XO, 17% triploid and 6% tetraploid. The frequency with which an individual chromosome was found to be trisomic varied. The highest frequency was that of trisomy 16 which formed 31% of the total trisomics. Next was trisomy 22 forming 11%, and trisomy 21 forming 9% of the total trisomics. No cases of trisomy 1 were recorded and all the large chromosomes had low frequencies of trisomy.

**Estimated frequency of aneuploidy at conception (1975)**

Boué et al. estimated the frequency of aneuploidy at conception to be 50%. To reach this estimate they used their figure of 60% of spontaneous abortions having a chromosome anomaly and assumed that all chromosomes had an equal frequency of aneuploidy. They took 15% monosomy X and 15% trisomy 16 as being typical for all chromosomes. Their estimate of frequency of aneuploidy is likely to be an overestimate because as pointed out by Carr and Gedeon (1977) the 60% estimate of aneuploidy in spontaneous abortions is likely to be an overestimate and secondly, there is no good evidence that each chromosome has the same frequency of aneuploidy.

Alberman and Creasy (1977) estimated that, of diagnosed pregnancies, approximately 8% were chromosomally abnormal at conception. This estimate was based on the percentages of chromosome anomalies found among spontaneous abortions, stillborns, neonatal deaths and liveborns. The percentage of chromosome anomalies among undiagnosed pregnancies was not considered. For this reason 8% is likely to be an underestimate of the frequency of aneuploidy at conception.
Attempts have been made to estimate the frequency of aneuploidy at conception by examination of human sperm. Assuming that aneuploid sperm are equally effective in fertilizing ova this would give some indication of the male contribution to aneuploid conceptions. Pearson and Bobrow (1970) and Sumner et al. (1971) attempted to measure aneuploidy using the F-body method in which the presence of two fluorescent bodies was assumed to identify sperm with two Y chromosomes. This method gave estimates of 0.9 - 1.4% for the Y chromosome and, assuming equal frequencies for all chromosomes, the estimate of the frequency of aneuploid sperm was over 20%. This method has been severely criticised by Sumner and Robinson (1976) and Beatty (1977, 1978) on the grounds that many of the extra F-bodies were not Y chromosomes. Also the estimates of aneuploidy obtained seemed intuitively too high.

A more interesting method has been described by Rudak et al. (1978) in which the chromosome complement of human sperm is visualised by using them to fertilize hamster oocytes. Once fertilization has occurred the sperm nucleus is reactivated and the chromosomes condense. Karyotype analysis can then be carried out. This technique has been used by Rudak et al. (1978) and Martin et al. (1982) to determine the frequency of numerical anomalies in human sperm. They obtained estimates of 5% and 10% respectively. If equivalent anomalies occurred in the female then the frequency of aneuploidy at conception would be 10 - 20%. However, as the age effect in aneuploidy is only observed
to any great extent in the female it is uncertain whether there is an equal risk of non-disjunction in men and women.

It is clear that the estimate of the real frequency of aneuploidy at conception is fraught with difficulties brought about by the unknown frequency in early pregnancies. However, even the lowest estimates are unusually high when compared to those in laboratory and domestic animals presented in Table 1.1 (page 12).

We have therefore a range of estimates of the frequency of numerical chromosome anomalies in man. The frequency is greater the earlier in pregnancy a survey is carried out. At all stages of investigation, trisomics form the major class of numerical anomalies. Rarely cases of monosomy G have been reported (Sankaranarayanan, 1979) but no other autosomal monosomics. This indicates that an extra chromosome is more readily tolerated than a missing one, as for every trisomy generated at meiosis by non-disjunction there should be a monosomy. The deficiency of autosomal monosomics could have many explanations. Nullisomic gametes may be unable to take part in fertilization. The deficiency of gene products, from loci on the missing chromosome, may be lethal, or lack of a chromosome may allow expression of harmful recessive genes. This could lead to early embryonic death and elimination of the zygote before pregnancy has been recognized.

Ford and Evans (1973) showed that in mice chromosomally unbalanced sperm could fertilise eggs and in Drosophila gametes with only chromosome 4 would appear to be functional.
The hypothesis of early embryonic death of monosomics has been confirmed for mice by Gropp (1973) using stocks with Robertsonian translocations. Stocks heterozygous for such translocations have high frequencies of disomic and nullisomic gametes. Examination of preimplantation embryos showed a bimodal distribution of hypoploidy and hyperploidy while early post-implantation embryos showed virtually no hypoploidy.

At later stages of development sex chromosomal anomalies form the major class of numerical anomalies, for example trisomy X forms 9.6% of trisomics among liveborns compared to 0.5% among spontaneous abortions. This is consistent with the less severe effects of sex chromosomal aneuploidy noted in liveborns, compared to the effects of autosomal aneuploidy. There is an excess of XO individuals over trisomy X, suggesting that XO may arise through errors other than those producing a coincident trisomy. There is some evidence to suggest that XO individuals arise not through meiotic errors but through post-fertilization chromosome loss. Russell and Saylors (1960) showed increased frequencies of XO mice following irradiation after fertilization.

As pointed out earlier, the frequency of aneuploidy at birth is the most important when considering the consequences for society. Approximately four in every one thousand births have a numerical chromosome anomaly, the majority of these being sex chromosomal aneuploidy or trisomy 21. As all these are associated with mental
retardation this places a high financial load on society as well as causing much distress. The high frequency of spontaneous abortions, many of which are due to aneuploidy, also causes distress.

Man appears to be unusual in having this high frequency of aneuploidy as can be seen from consideration of aneuploidy in other mammals.

2b. The incidence of aneuploidy in other mammals

Table 1.1 gives some examples of the incidence of aneuploidy in other mammals. A more comprehensive account of mouse and human data can be found in Nijhoff and de Boer (1981). As estimates of monosomy and nullisomy are often subject to error due to technical difficulties, the incidence of aneuploidy is generally taken to be twice the incidence of trisomy or disomy. It can be seen from the table that estimates are very variable. Part of this variation can probably be attributed to the small number of hyperploids found, with most of the estimates quoted being based on just one observation. Despite this variation it is clear that at all stages, the incidence of aneuploidy in other mammals is much less than that in man. Even the estimate of Golbus (1981) of 6.18% disomy in metaphase II oocytes, which is exceptionally high compared to all other mouse data, only approaches the lowest estimate of aneuploidy at the equivalent stage in man.

Although the majority of data on incidence of aneuploidy is from the mouse there is sufficient information from other organisms to indicate that it is man, and not
TABLE 1.1a. The incidence of aneuploidy in mammals other than man at haploid stages

<table>
<thead>
<tr>
<th>STAGE STUDIED</th>
<th>SPECIES</th>
<th>n-1 %</th>
<th>n+1 %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metaphase II oocytes</td>
<td>Mouse (CFLP)</td>
<td>7.8</td>
<td>0.26 *</td>
<td>Polani &amp; Jagiello(1976)</td>
</tr>
<tr>
<td>(matured in vitro</td>
<td>&quot; (CS1)</td>
<td>7.75</td>
<td>0.27 *</td>
<td>&quot;</td>
</tr>
<tr>
<td>unless stated)</td>
<td>&quot; (Q)</td>
<td>N.G.</td>
<td>1.7</td>
<td>Speed (1977)</td>
</tr>
<tr>
<td>&quot; (C3H/HeHan)</td>
<td>N.G.</td>
<td>0.24 *</td>
<td>Hansmann &amp; El-Nahass(1979)</td>
<td></td>
</tr>
<tr>
<td>&quot; (101xC3H)F1</td>
<td>N.G.</td>
<td>0.30 *</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>&quot; (NMRL/Han)</td>
<td>N.G.</td>
<td>0.23  *</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>in vivo</td>
<td>&quot; (Swiss Webster)</td>
<td>20</td>
<td>1.14</td>
<td>Golbus (1981)</td>
</tr>
<tr>
<td>&quot; (CBA)</td>
<td>5.78</td>
<td>2.44</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>in vivo</td>
<td>&quot; (CBA)</td>
<td>6.18</td>
<td>0.73</td>
<td>&quot;</td>
</tr>
<tr>
<td>Chinese hamster</td>
<td>N.G.</td>
<td>0</td>
<td>Hansmann &amp; Probeck(1979)</td>
<td></td>
</tr>
<tr>
<td>Syrian hamster</td>
<td>N.G.</td>
<td>0.33  *</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>Metaphase II spermatocytes</td>
<td>Mouse (Various)</td>
<td>1.3</td>
<td>0.19</td>
<td>Beatty et al.(1975)</td>
</tr>
<tr>
<td></td>
<td>&quot; (CFLP)</td>
<td>4.6</td>
<td>0.26 *</td>
<td>Polani &amp; Jagiello(1976)</td>
</tr>
<tr>
<td></td>
<td>&quot; (CS1)</td>
<td>8.84</td>
<td>0.87</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>&quot; (C57BL)</td>
<td>N.G.</td>
<td>0.45 *</td>
<td>Speed (1977)</td>
</tr>
<tr>
<td></td>
<td>&quot; (CBA)</td>
<td>N.G.</td>
<td>1.00 *</td>
<td>&quot;</td>
</tr>
<tr>
<td>Chinese hamster</td>
<td>1.79</td>
<td>0.26 *</td>
<td>Hultén et al.(1970)</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>0</td>
<td>0</td>
<td>Eliasson et al.(1967)</td>
<td></td>
</tr>
<tr>
<td>Spermatids</td>
<td>Field vole</td>
<td>N.G.</td>
<td>0.07 **</td>
<td>Tates (1979)</td>
</tr>
</tbody>
</table>

N.G. Not Given
* Based on a single observation
** Only sex chromosomes studied.
**TABLE 1.1b.** The incidence of aneuploid in mammals other than man at diploid stages.

<table>
<thead>
<tr>
<th>STAGE STUDIED</th>
<th>SPECIES</th>
<th>n-1 %</th>
<th>n+1 %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st cleavage division</td>
<td>Mouse (CF1)</td>
<td>1.8</td>
<td>0</td>
<td>Donahue (1972)</td>
</tr>
<tr>
<td></td>
<td>&quot; (CFLP)</td>
<td>8.3</td>
<td>0.52*</td>
<td>Kaufman (1973)</td>
</tr>
<tr>
<td></td>
<td>&quot; (Various)</td>
<td>0.5-2.1</td>
<td>0.5-1.2</td>
<td>Maudlin &amp; Fraser (1978a,b)</td>
</tr>
<tr>
<td>pre-implantation embryos</td>
<td>Mouse (CBA/HT6)</td>
<td>N.G.</td>
<td>0.9*</td>
<td>Gosden (1973)</td>
</tr>
<tr>
<td></td>
<td>Chinese hamster &quot; &quot;</td>
<td>0.44</td>
<td>0.44*</td>
<td>Binkert &amp; Schmid (1977)</td>
</tr>
<tr>
<td></td>
<td>Rabbit &quot; &quot;</td>
<td>N.G.</td>
<td>0.3*</td>
<td>Yamamoto &amp; Ingalls (1972)</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>0</td>
<td>0.9</td>
<td>Fechheimer &amp; Beatty (1975)</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>4.5</td>
<td>4.5</td>
<td>Shaver (1973)</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>0</td>
<td>0</td>
<td>McFeely (1967)</td>
</tr>
<tr>
<td>post-implantation embryos</td>
<td>Mouse (CBA)</td>
<td>0</td>
<td>0.94</td>
<td>Long &amp; Williams (1980)</td>
</tr>
<tr>
<td></td>
<td>&quot; (Q)</td>
<td>0</td>
<td>0.36</td>
<td>Yamamoto &amp; Ingalls (1972)</td>
</tr>
<tr>
<td></td>
<td>Syrian hamster</td>
<td>2.3</td>
<td>0</td>
<td>Chandley &amp; Speed (1979)</td>
</tr>
<tr>
<td>Liveoorns</td>
<td>Mouse (Balb c x Balb c/129) &quot; ?</td>
<td>0</td>
<td>0</td>
<td>Goodlin (1965)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25-0.35**</td>
<td>0.01**</td>
<td>Russell (1979)</td>
</tr>
</tbody>
</table>

N.G. Not Given

* Based on a single observation

** Only sex chromosomes studied.
the mouse, which is the exception. Unfortunately no published data is available for comparison with other primates but there is some indication that these too may have a high incidence of aneuploidy. Bobrow (unpublished, quoted Bond and Chandley, 1983) in work on the marmoset, Callithrix jacchus, says there is some evidence that foetal loss due to chromosome abnormalities has a similar frequency to that in man. More data is needed to decide whether a high frequency of aneuploidy is peculiar to primates or to man.

One explanation suggested for the lower frequency observed in other mammals is that, in these, aneuploidy interferes with the functioning of the gametes and prevents chromosomally abnormal gametes being involved in conception. This explanation is ruled out by the low incidence observed in metaphase II oocytes. Whatever the reason, there is no doubt that the incidence of aneuploidy in man is high and that although a large proportion of aneuploid foetuses do not survive to term, a considerable number do.

3. Concern about environmental agents inducing aneuploidy

In recent years there has been concern expressed that environmental agents, both chemical and radiation, may increase the frequency of aneuploidy in man. Examples causing concern include the reports of increased trisomy 21 among offspring of younger mothers (Nordenson, 1979 and Koulischer and Gillerot, 1981). The latter workers found the new pattern to be most pronounced in the large industrial region of Charleroi, Belgium, and suggested that there may be an environmental component. Two possibilities were
the high concentration of industry and the greater availability of oral contraceptives. Harlap et al. (1980) surveyed spontaneous abortions in women with a known contraceptive history. They found a small increase in spontaneous abortion associated with use of oral contraceptives after the last menstrual period, but this increase was not statistically significant. They concluded that their sample was not sufficiently large to say there was no risk when using oral contraceptives, but that any risk would be small. An oral contraceptive involves long term maintenance of abnormal conditions in a healthy person, and has the direct consequence of altering the gametic environment. Investigations as to whether such a compound may increase aneuploid conceptions should not be left to retrospective studies. A preclinical testing system should be available. Although contraceptives are an obvious example the continual development of new pharmaceutical compounds and industrial chemicals in our society suggest an increased risk of environmental factors influencing aneuploidy.

Another reason for concern is that despite the amniocentesis screening programme, now available to older women for the detection of aneuploid foetuses, the incidence of Down's syndrome births has remained fairly constant, again suggesting an increase in births to younger mothers. Mikkelsen et al. (1976) report that although maternal age has decreased in their study area, the incidence of aneuploidy has remained fairly constant over twelve years. The very high incidence of aneuploidy in man could mean
that human meioses are vulnerable to induction of aneuploidy.

Already great care is taken to screen for mutagens which could increase the frequency of point mutations and chromosome breakage. However, none of the assay systems currently in use can detect agents whose sole genetic end point is to induce aneuploidy (de Serres, 1979). Bellincampi et al. (1980) carried out tests for both point mutations and mitotic non-disjunction and found a selection of chemical agents which did not induce point mutations but did induce non-disjunction. There is less risk of an aneuploid defect being passed on to future generations than a point mutation, due to the shortened lifespan, mental retardation and sterility often associated with aneuploidy. Nevertheless the severe effects and the already high incidence which occur every generation mean it is desirable to prevent any increase in aneuploidy. Much work is currently being put into the development of new systems to allow testing of the effects of environmental agents on aneuploidy levels.

Testing systems will fall into two main categories; those using lower eukaryotes which will allow rapid, inexpensive detection of possible inducers and so be suitable for mass screening and those using mammals which would provide a data base from which to extrapolate to man in order to make risk estimates. The large differences in both cellular organisation and cell division between prokaryotes and eukaryotes mean that assay systems should use eukaryotes. The problems of using lower eukaryotes will be considered later.
Meiosis is a complex process involving coordination of many different steps. It can be separated into two major divisions: the first (reductional) division and the second (equational) division which is analogous to a mitotic division. These are themselves brought about by a series of events and faults in any one of these can lead to a defective meiosis, one possible outcome being aneuploidy. Table 1.2 indicates some of these errors and emphasises the number of possible origins of aneuploidy. Ideally a testing system would allow various types of error to be distinguished.

4. **Meiotic errors resulting in aneuploidy: the occurrence in man and other organisms**

a. **First division non-conjunction and non-disjunction.**

First division non-disjunction, with paired homologues failing to separate and so travelling to the same pole at the first meiotic division, was one of the first mechanisms of abnormal chromosome segregation to be suggested (Bridges, 1913). Further work by Bridges (1916) gave detailed accounts of proposed first division non-disjunction of the sex chromosomes of *Drosophila ampelophila*. However, as pointed out by Sturtevant and Beadle (1939), the same result could be achieved by non-conjunction, that is pairing failure of the homologues and both passing to the same pole at the first meiotic division. These two errors can only be distinguished genetically by the fact that trisomy arising through non-conjunction would not be expected to have any recombination along the chromosome involved. Non-
TABLE 1.2. Events in meiosis where errors could result in aneuploidy.

<table>
<thead>
<tr>
<th>Meiotic Division</th>
<th>Event</th>
<th>Possible errors giving rise to aneuploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre- 1st division</td>
<td>DNA synthesis</td>
<td>Extrareplication of chromosomes</td>
</tr>
<tr>
<td>I</td>
<td>Chromosome pairing</td>
<td>Abnormal synaptonemal complex causing reduced pairing</td>
</tr>
<tr>
<td>I</td>
<td>Chiasma formation and recombination</td>
<td>Increased levels preventing normal separation of chromosomes. Decreased levels causing failure to pair sufficiently.</td>
</tr>
<tr>
<td>I</td>
<td>Structural re-arrangements of chromosomes</td>
<td>Abnormal associations of chromosome material resulting in unequal distribution between daughters.</td>
</tr>
<tr>
<td>I/II</td>
<td>Chromosome attachment to the spindle</td>
<td>Abnormal spindle or chromosomes preventing normal interaction</td>
</tr>
<tr>
<td>I/II</td>
<td>Centromere division</td>
<td>Premature division at 1st division Premature division at 2nd division Failure to divide at 2nd division</td>
</tr>
<tr>
<td>I/II</td>
<td>Separation of homologues or sister chromatids</td>
<td>Abnormal spindle resulting in failure to divide equally between poles.</td>
</tr>
</tbody>
</table>
disjunction, on the other hand, would be expected to exhibit normal, or even increased, frequencies of crossing-over. Because crossing-over is not normally monitored most analyses of first division errors do not distinguish non-conjunction and non-disjunction.

The occurrence of first division errors has been confirmed in fungi, for example in *Neurospora crassa* by Threlkeld and Stephens (1966) and Threlkeld and Stoltz (1970), in *Sordaria brevicollis* by Bond (1976) and in yeast by Parry et al. (1979a) and Sora et al. (1982).

Merriam and Frost (1964) investigated aneuploidy in *Drosophila melanogaster* and attempted to distinguish non-conjunction and non-disjunction by looking at exchange in the X chromosomes of progeny with an aneuploid complement of sex chromosomes. They used the algebraic methods of Weinstein (1936) to calculate the expected proportions of meioses with 0, 1, 2, 3 and more crossovers along the X chromosome. This method assumes no detectable exchanges are missed and that there is random involvement of sister chromatids. From this the number of undetectable exchanges can be estimated. They found a bimodal distribution of exchange in aneuploid progeny with larger numbers of zero and two exchange chromosomes, compared to single exchanges, than expected. The lack of clustering of exceptional progeny in broods showed that the exceptions arose through meiotic and not premeiotic events. The excess of zero exchanges suggested that non-conjunction could be occurring. The existence of aneuploid flies with cross-overs on the X
chromosome confirmed that non-conjunction was not the sole source of aneuploid progeny. Some specially constructed compound X stocks are known which show an excess of zero exchanges, with no evidence of asynapsis. Merriam and Frost (1964) therefore pointed out that their observed excess of zero exchanges did not necessarily imply non-conjunction.

Among laboratory mammals, most work on aneuploidy has been carried out in mice. First division errors have been detected through chromosome counts at metaphase II and many studies have shown that these errors do occur (see Table 1.1, page 12). They have also been detected in Syrian hamsters (Hansmann and Probeck, 1979).

In man it is now well established that the majority of trisomy, at least for chromosome 21, results from maternal first division errors, although there is also a paternal component (Licznerski and Lindstein, 1972; Langenbeck et al., 1976; Jacobs and Morton, 1977 and Hansson and Mikkelsen, 1978). Distinguishing first division errors in man relies on chromosome polymorphisms giving information about when and in which parent the defect originated. If it is possible to distinguish chromosomes of the parents, and if a parent is heterozygous for a polymorphism then, if a trisomic child carries both homologues from that parent, it can be assumed a first division error occurred in that parent.

b. **Premature centromere division at the first meiotic division**

Premature centromere division, precocious centromere division, predivision and presegregation are all terms used
to describe the phenomena of early separation of sister chromatids, at meiosis or mitosis. In the ascomycete fungi premature centromere division at the first meiotic division can be distinguished from other first division errors because only half of the products from a single meiosis are aneuploid. Threlkeld and Stoltz (1970) identified ascus types in *Neurospora crassa* which were consistent with premature centromere division at the first meiotic division. Campbell et al. (1975) suggested premature centromere division as a means of disomic yeast cells losing a chromosome and regaining the haploid state. This would, however, be at a mitotic division.

Premature centromere division has been described in mutants of maize and *Drosophila*. These cases will be considered in a later section dealing specifically with mutants which lead to increased frequencies of aneuploidy.

In the mouse, Polani and Jagiello (1976) have suggested that the occurrence of univalents at metaphase I may lead to 'predivision' of one or both of the unpaired centromeres. Hansmann and El-Nahass (1979) suggested 'presegregation' at meiosis I to explain their observations of metaphase II oocytes with 19 chromosomes plus 1 chromatid or 20 chromosomes plus 1 chromatid (n = 20).

Premature centromere division at the first meiotic division can be detected either by cytological examination of gametes or by the order of meiotic products in a tetrad. As human aneuploidy data are based on examination of the aneuploid individual, not of meiotic products, premature
centromere division can not be distinguished from other first division errors. It has been postulated that premature centromere division does occur at mitosis in man, and that the frequency increases with age. Fitzgerald (1975) suggested premature centromere division to explain the presence of XO cells in lymphocyte cultures, the frequency of which increase, with increasing age of the subjects. Galloway and Buckton (1978) reported an age-related increase in hypoploidy of the X-chromosome and also of some autosomes. Hyperploidy increased too, but to a lesser degree. It is unclear whether premature centromere division occurs at the first meiotic division in man.

c. **Second division non-disjunction**

Non-disjunction at the second meiotic division can be distinguished from first division errors by the use of centromere markers or chromosome polymorphisms, provided that these do not recombine with the centromere. Disomic products which arise through second division errors will be homozygous for centromere markers. First division errors, on the other hand, will result in disomic products heterozygous for centromere markers. Second division non-disjunction can arise through failure of the chromatids to separate at the second meiotic division. The same chromosome complement can also arise if there is premature separation of the chromatids at the second meiotic division and both chromatids pass to the same pole at anaphase II. Either of these errors will result in a hyperploid product which will be homozygous for any centromere marker.
Second division errors are commonly described as second division non-disjunction.

Threlkeld and Stoltz (1970) detected a low frequency of asci of *Neurospora crassa* which could be attributed to second division errors and the same has been found by Bond (1976) in *Sordaria brevicollis*. In both cases only a fraction of the aberrant divisions could be detected, as crossing-over in a restricted interval was necessary for the error to be revealed. Sora et al. (1982) reported the detection of second division errors in yeast.

Bridges (1916) proposed second division non-disjunction to explain some of the exceptional progeny among his *Drosophila*. Once the position of the centromere of the X chromosome was mapped (Anderson, 1926), this explanation proved incorrect (Moriwaki, 1938). Merriam and Frost (1964), using multiply marked X chromosomes found no evidence for second division non-disjunction in *Drosophila*.

Mice with an XYY chromosome constitution have been described by Cattanach and Pollard (1969). An XYY individual probably arose following second division non-disjunction in the male. An XYY chromosome constitution could also arise if mitotic non-disjunction occurred soon after fertilization. This could produce an XO/XXY mosaicism which may go undetected.

Rodman (1971) suggested precocious centromere separation at metaphase II leading to second division non-disjunction in mouse oocytes.

Second division errors in man have been described in
anecdotal reports, for example Juberg and Jones (1970). In this case the mother had one chromosome 21 with short arms, the other being normal, the father had two normal chromosomes 21s and the child had two with short arms and one normal chromosome 21. The detection of XYY men, first reported by Sandberg et al. (1961), also indicated that second division errors may occur in man. As described above, it is always possible that mitotic non-disjunction and second division non-disjunction could be confused if the mosaicism arising through mitotic non-disjunction went undetected.

The development of chromosome banding techniques, revealed banding polymorphisms, as well as the more obvious structural polymorphisms. This allowed more extensive investigations of the frequencies of aneuploidy due to second division errors in man and several such studies have been carried out (Licznerski and Lindstein, 1972; Mikkelsen et al., 1976; Wagenbichler et al., 1976). All these studies reported only the fully informative families in their samples. Langenbeck et al. (1976) and Jacobs and Morton (1977) pointed out that this method of selecting data biased the analyses, resulting in overestimates of the frequency of second division non-disjunction. The maximum likelihood analysis of Jacobs and Morton (1977) included all the information and so gave a more accurate figure. Their estimate of 29% of aneuploidy detected in man being due to second division non-disjunction is much lower than the 38% estimate of Mikkelsen et al. (1976).
It seems clear that, although the frequency is uncertain, second division non-disjunction does occur in man.

d. **Extrareplication of chromosomes**

All the meiotic errors described above are errors in the distribution of chromosomes at cell division, resulting in equal numbers of hyperploid and hypoploid products. Aneuploidy can also arise through errors in DNA replication prior to meiosis. In this case the chromosome number is abnormal before the cell division starts, and no hypoploidy will occur. Endoreduplication, or an extra round of DNA replication for the complete chromosome set, results in diploid gametes. This has been reported in cell culture of human lymphocytes (Takanari and Izutsu, 1981) and of Chinese hamster cells (Rizzoni and Palitti, 1973). Diploid gametes have been found in mice (Maudlin and Fraser, 1978a) and the occurrence of triploidy in man (Boué et al., 1975; Jacobs and Morton, 1977) indicates diploid gametes exist. Whether these arise through failure of meiosis or endoreduplication is unclear. Endoreduplication will result in polyploidy, not aneuploidy.

Extrareplication of one or a few chromosomes, to generate aneuploidy, has only been recorded in the fungi. Moustacchi et al. (1967) reported a mutant, AM, in yeast which generated aneuploidy because the chromosomes had a tendency to undergo extrareplication. Many chromosomes were affected and near diploids were produced. Extrareplication was suggested by Case and Giles (1964) and
Threlkeld and Stephens (1966) as a possible origin of asci of *Neurospora crassa*, containing pseudowild type but no abortive spores. Bond (1976) reported recovery of an extra chromosome from pseudowild type spores of *Sordaria brevicollis*, which were unaccompanied by abortive spores in the ascus. Another possible explanation is that mitotic non-disjunction had occurred prior to meiosis, so an extra chromosome is already present when a cell enters meiosis.

Both events are difficult to detect unless all meiotic products are examined, as their occurrence is inferred from the observation of chromosome gain without corresponding chromosome loss. As only individual products of meiosis are examined in man, it is possible such errors would be attributed to errors at the first or second division of meiosis.

e. Chromosome rearrangements

All the errors which have been discussed cause primary trisomy or monosomy. However, secondary and tertiary aneuploidy are also found. These are defined below and illustrated in Figure 1.1. In *primary trisomy* the extra chromosome is structurally normal. This is not true of secondary and tertiary trisomy. In *secondary trisomy* the extra chromosome is an isochromosome for one chromosome arm. Essentially the organism will have four copies of this arm but a normal complement of all other chromosome arms. In *tertiary trisomy* the extra material is involved in a translocation.
FIGURE 11 Illustration of primary, secondary and tertiary aneuploidy

a) Normal diploid

b) Primary trisomy for chromosome I

d) Secondary trisomy for chromosome I

d) Tertiary trisomy
The descriptions secondary and tertiary therefore indicate that the aneuploidy has arisen through structural rearrangements of the chromosomes. The errors giving rise to such aneuploidy will be different from those inducing primary trisomy and monosomy and will involve chromosome breakage and reunion. While such aneuploidy is important (0.26% of live born infants have structural chromosome anomalies (Hook and Hamerton, 1977)) the main emphasis in this thesis will be on primary aneuploidy. Agents inducing chromosome rearrangements are likely to be detected in other mutagenic testing systems, as their effect involves interaction with the DNA. Some consideration will be given to tertiary effects in a later chapter dealing with X-ray induced aneuploidy.

f. Mutants which give rise to increased frequencies of aneuploidy

Mutants which exhibit a greatly increased frequency of meiotic errors are generally known as meiotic mutants. General descriptions of the genetic control of meiosis can be found in reviews by Baker et al. (1976) and Golubovskaya (1979). A brief description of some meiotic mutants will be given here to illustrate some errors which result in aneuploidy.

Fungi. Smith (1975) analysed a recessive meiotic mutant, mei-1, which affected all linkage groups of Neurospora crassa. Reduced crossing over caused extensive aneuploidy. The normal synaptonemal complex was absent. The dominant
mutation, Mei-1, also had reduced recombination and enhanced aneuploidy. Delange and Griffiths (1980a,b) found two meiotic mutants, asc(DL879) and asc(DL95) which reduced recombination and increased first division errors in *N. crassa*. Cytological examination revealed reduced pairing of homologues. Meiosis II was also irregular and ascus types consistent with both first and second division errors were observed. The mutant asc(DL243) increased non-disjunction at the second meiotic division and in the post-meiotic cell divisions (Delange and Griffiths, 1980b). The association between centromeres and one of the spindle pole bodies was defective, but it was not clear which component was altered. Another mutant which affected the second meiotic division and the next mitosis was mei-4, in which chromosomes failed to separate clearly at these divisions (Perkins and Barry, 1977). Zickler et al. (1982) reported two genes in *Sordaria macrospora* which affected chromosome disjunction at both meiotic divisions. These mutants, spo44 and spo77, had abnormal synaptonemal complexes.

Maize. Maguire (1978a,b) has studied premature centromere division and the synaptonemal complex in maize and reported premature centromere division at the first meiotic division in asynaptic mutants. Both divisions showed this phenomenon in desynaptic mutants. Further work, (Maguire, 1982) using pericentric inversions to alter pairing, showed that asynapsis at meiosis I could result in lagging monads at anaphase II. She suggested that one function of the synaptonemal complex
may be to promote sister centromere association until anaphase II.

**Drosophila.** There are many meiotic mutants known in *Drosophila melanogaster* which affect chromosome disjunction. These have been discussed by Baker *et al.* (1976) and Golubovskaya (1979). The recessive mutant, *mei-1*, decreased recombination along the X chromosome and trebled non-disjunction of the X chromosome in females. There were also twenty other female specific loci which decreased recombination in all chromosomes. Flies mutant at these loci exhibited increased frequencies of aneuploidy arising at the first meiotic division. Only non-exchange chromosomes were involved in these errors suggesting that non-conjunction or incomplete pairing caused the problems at division. Some of these mutants were asynaptic (*c(3)G, mei-W68*) while others had normal synaptonemal complexes (*mei-9a, mei-218, mei-41*).

The *nod* mutation had no effect on cross-over frequency but did increase aberrant segregation of non-exchange chromosomes. Yet another class of mutant affected both exchange and non-exchange chromosomes at the first meiotic division in females. The largest effect was on chromosomes X and 4. The second meiotic division was not affected. It was suggested that these mutants (*ca* and *1(1) TW-6 CS* in *Drosophila melanogaster* and *ca* in *D. simulans*) affected either the spindle or the chromosome attachment site. Baker *et al.* (1976) were not in favour of a general effect on the spindle as the second meiotic division was not affected.
There are also male specific meiotic mutants in *Drosophila* which act on the first meiotic division and are chromosome specific. There are twenty X linked mutations affecting only X-Y disjunction. *mei-58* acted on chromosome 4 alone and *mei-G17* acted on chromosome 2 and the sex chromosomes. Chromosomes 1, 2 and 4 showed increased aneuploidy under the influence of *mei-O81*, and the mutant *pal* caused loss of any chromosome.

Although spontaneous second division non-disjunction has not been detected in *Drosophila* meiotic mutants which affect this division have been described (Baker et al., 1976). The *mei-G17* mutation, on chromosome 2, increased second division aneuploidy of all chromosomes in the male. The *eq* mutation caused second division non-disjunction of the X chromosome in both sexes and *mei-G87* had this effect on chromosome 2. Davis (1971) described a meiotic mutant *mei-S332* which affected both sexes and resulted in a high frequency of second division errors and some first division errors. This mutant caused premature separation of sister chromatids. If this occurred at the second meiotic division and the unpaired sister chromatids travelled to the same pole, a disomic gamete would be formed. If the premature separation occurred at the first meiotic division a disomic gamete would arise if sister chromatids moved to opposite poles.

Although pairing failure is well documented among the mutants mentioned above, this may not contribute to chromosomal abnormalities in human gametes. There is evidence
that extensive pairing failure in spermatogenesis results in germ cell arrest in man and mouse (Pearson et al., 1970; Beechey, 1973; Purnell, 1973; Chandley et al., 1976). It is not known whether this is true of oogenesis.

Man. Evidence for meiotic mutants, which cause aneuploidy in man, is slim. This is probably due to limited pedigree data. Baker et al. (1976) gave a brief summary of the evidence for non-randomness of chromosomal abnormalities. Several cases have been reported of families where two different chromosomal anomalies have occurred. The same sex chromosome anomaly was found in sibs, more often than would be expected by chance. In the families of 60 trisomy D or trisomy 18 individuals there were three cases of sibs and one case of an uncle with Down's syndrome. However, non-random clustering could also be due to environmental effects and there is also the problem of bias of ascertainment of cases of aneuploidy.

Michels et al. (1982) reported chromosome translocations occurred at a greater frequency among parents with multiple spontaneous abortions than in the general population. Also over 1% of the parents studied were XO mosaics. Hecht (1982) suggested that XO mosaicism may be indicative of impaired control of chromosome disjunction and hence of an increased risk of producing aneuploid offspring. Chaganti et al. (1980) looked at male infertility and azoospermia of sibs in a family with related parents, and noticed asynapsis in infertile males. They suggested that an autosomal recessive was being expressed both in this case and in other
cases of asynapsis in man where there was parental consanguinity or affected sibs.

Alfi et al. (1980) studied Down's syndrome in a highly inbred population in Kuwait. Forty percent of marriages were closely consanguineous. They found that the risk of having a Down's syndrome child was four times greater in consanguineous marriages. This observation, and the fact that parents who are consanguineous have themselves a higher probability of being offspring of consanguineous marriages, led the authors to suggest there may be a gene or genes segregating which increased the probability of non-disjunction at meiosis.

Seven genetic conditions are known in man which affect the chromosome stability at mitosis, but no detailed meiotic studies are available. These conditions are Bloom's syndrome, Fanconi's anaemia, Louis-Bar syndrome, xeroderma pigmentosum, incontinentia pigmenti, porokeratosis of Mibelli and Robert's syndrome (Baker et al., 1976 and German, 1979). Cytogenetical analysis of cells from patients with Robert's syndrome showed that there was premature separation of centromeres at mitosis. This was interpreted as a possible genetically determined change causing disruption of chromosome segregation (German, 1979). Although all these conditions have similar effects on mitosis, the conditions vary in many ways, and each may be genetically heterogeneous.

Testing Systems

It is clear, then, that aneuploidy is not the result of one particular meiotic error, but can arise from a range
of disturbances of cell division. It is possible that all these errors may occur in man. A satisfactory testing system should therefore be able to detect as wide a range of errors as possible. Ideally a system should be suitable for obtaining risk estimates and for mass screening of potential aneuploid inducers. Various systems have been developed using mammals, Drosophila and fungi and these are discussed below.

a) Mammalian Testing Systems

Several methods have been described to use mammals in testing for the induction of aneuploidy. These vary in the stage of meiosis, or the embryonic age at which the aneuploid levels are determined. Methods for detecting aneuploidy in human sperm are obviously not intended for use as aneuploid screening methods. Rather, the purpose of such systems is to allow retrospective studies, to check whether any section of the population may have been put at risk through exposure to an environmental agent. These techniques have been reported while considering the incidence of aneuploidy in human gametes and will not be discussed further here. Other mammalian systems will be considered according to the stage of meiosis or the embryonic age studied. The majority are based on the mouse, but some other organisms have been used.

Analysis at metaphase I

The frequency of univalents at metaphase I has been suggested as a means of determining frequencies of aneuploidy.
Polani and Jagiello (1976) and Speed (1977) found no parallel between the frequency of univalents at metaphase I and identifiable chromosome errors at metaphase II in either male or female mice. This is not, therefore, a satisfactory method of estimating frequencies of aneuploidy.

**Analysis at metaphase II**

This has been carried out on metaphase II oocytes or spermatocytes. Oocytes are obtained following superovulation and are either cultured *in vitro* or *in vivo*. No banding technique is available to distinguish different chromosomes but staining of constitutive heterochromatin was used (Nijhoff and de Boer, 1981). As hypoploidy can arise artefactually, due to chromosome loss during preparation, aneuploid levels are usually given as twice the incidence of hyperploidy. Many workers have used this technique to examine frequencies of aneuploidy following various treatments, for example Röhrborn and Hansmann (1976) and Martin et al. (1976), using mouse oocytes and also references in Table 1.1. Gates et al. (1981) suggested using prepubertal mice as the source of oocytes, arguing that this gave consistently high yields of oocytes on superovulation and that using three week old mice lowered animal costs. They found no cases of spontaneous hyperploidy. The suitability of using prepubertal mice in a screening system depends on their susceptibility to induction, which has not yet been shown.

It has been demonstrated that the use of hormones to cause superovulation did not affect chromosome disjunction
at meiosis I (Hansmann and El-Nahass, 1979) but it should be borne in mind that such treatment may alter the sensitivity of oocytes to environmental agents. Tease (1982) investigated the response to X-irradiation following varying doses of hormones, but found no differences in sensitivity. No investigation of this kind has been carried out using chemical agents.

Metaphase II spermatocytes have been used by Beatty et al. (1975), Polani and Jagiello (1976) and Speed (1977). Analysis of males has the disadvantage that well spread metaphases are more difficult to obtain than in females (Nijhoff and de Boer, 1981).

Analyses at metaphase II require separate preparations to be made to examine aneuploidy in both the male and the female and only first division errors can be detected. However an advantage is that treated mice do not have to be mated and females kept until the birth of offspring, in order to get results.

**Analysis of spermatids**

Tates (1979) studied sex-chromosome non-disjunction and diploidy in spermatids of the field vole, *Microtus oeconomus*. The X and Y chromosomes were distinguished from the autosomes by C-banding and XX, XY and YY sperm were recorded. Those with no identifiable sex chromosomes were not counted as nullisomics as some may have been due to staining failure. Diploids were distinguished by size.

This method, if reliable, would have the advantage
that both first division errors (XY sperm) and second division errors (XX and YY sperm) could be distinguished. However, the method is open to the same criticisms as those presented for the methods using F-bodies in human sperm and so may not be reliable.

Analysis at the first cleavage division

First cleavage division mouse embryos have been analysed to determine aneuploidy levels. Large numbers of eggs were obtained by superovulation and either in vivo or in vitro fertilization was used. In vitro fertilization did not affect the frequency of aneuploidy (Maudlin and Fraser, 1978a). It had the advantage that a higher proportion of scoreable chromosome spreads were obtained due to greater synchrony of fertilization. Maternal and paternal chromosomes could be distinguished due to the greater degree of condensation and of scatter of chromosomes found in the maternal set (Donahue, 1972 and Kaufman, 1973). The advantage of this method is that both male and female aneuploidy can be detected in one preparation and either or both parents can be treated. Errors at both meiotic divisions will be detected.

Analysis of pre-implantation embryos

Preimplantation embryos have been studied in many species, for example the mouse (Gosden, 1973), the rabbit (Fechheimer and Beatty, 1974; Shaver, 1973) and in hamsters (Yamamoto and Ingalls, 1972; Binkert and Schmid, 1977). Embryos are obtained following superovulation and in vivo
fertilization. This method has the advantage over studying later embryos in that embryonic material is very easy to obtain, with no contamination from maternal cells. Like first cleavage division studies errors at both meiotic divisions will be detected. Aneuploidy arising in either the male or the female can be detected, but not necessarily distinguished as maternal or paternal in origin.

Analysis of post-implantation embryos

Analysis of post-implantation embryos has been carried out on 9-10 day old embryos. Earlier post-implantation embryos could not be studied because of the problems of obtaining embryonic material uncontaminated with maternal cells. Most trisomic embryos were known to survive until day 10, although most monosomics were eliminated by this stage (Gropp, 1973).

This system has the advantage that good quality metaphase spreads are easily obtained, banding of chromosomes is possible and many foetuses can be analysed in a short time. A disadvantage is that information is lost compared to pre-implantation embryos as monosomics are not detected. Screening at this late stage is also more expensive as animals have to be maintained for longer periods.

Analysis of live borns

Genetic markers were used to identify aneuploidy of the sex chromosomes (Russell, 1979). Such methods are impractical as screening systems because large numbers of mice have to be maintained to detect any aneuploid
offspring. Lyon et al. (1976) described specially constructed stocks with Robertsonian translocations to detect autosomal aneuploidy among live born mice. This method has the disadvantage that both the male and female parents are chromosomally abnormal; therefore to use in screening would require treatment of abnormal stocks. Searle and Beechey (1982) discussed the possibility of developing a stock with several Robertsonian translocations which could be used to rescue aneuploid gametes from normal mice, so allowing treatments to be carried out on normal stocks. Although not yet developed this system has potential problems due to low fertility and small litter sizes in stocks with several Robertsonian translocations. Also aneuploid frequencies would be very difficult to determine as only aneuploid offspring would survive.

All the mammalian testing systems described require maintenance of considerable numbers of mice and are relatively time consuming due to the generation time of mice being nine weeks. Testing in the female is complicated by the fact that to screen all stages of meiosis would require treatment at many different times. Exposure while the females were in utero would be necessary to treat prediplotene stages. Diplotene stages would be exposed with treatment at birth. The treatment-conception interval following treatment in adult life would affect the stage of follicle development which was exposed. While mammalian testing systems are obviously necessary to obtain reasonable risk assessments they would not be
suitable for mass screening of potential inducers.

Different mammalian systems have different advantages as far as their use for risk assessment is concerned. Studies on live borns have the advantage of needing less technical expertise than cytological analyses, so reducing costs. However mice have to be maintained for longer periods and as aneuploid frequency is lower among live borns, more animals are needed to get reasonable numbers, in comparison to cytological analyses. The most useful cytological analyses are probably those which allow both male and female aneuploidy to be detected, that is, at first cleavage division and pre-implantation. As some aneuploids are lost by 10 days of gestation, post-implantation methods give less information.

What is clear is that for routine screening a more rapid and less expensive system is necessary.

b) Drosophila Testing Systems

The terminology used when discussing aneuploidy in Drosophila is slightly different from that used elsewhere. Primary exceptions have received two non-crossover homologues or two complementary cross-over homologues from the same parent. These must arise through first division errors. Equational exceptions have received a cross-over and a non-crossover homologue from a single parent and could be the result of first or second division non-disjunction. If the latter has occurred the homologue will be homozygous for proximal markers. If the error occurred at the first division the homologues will be homozygous
for distal markers.

Much work on non-disjunction in *Drosophila* has been carried out using marked sex chromosomes as this is a simple system with which to work. The aneuploids XXY, XYY and XO are viable and XXX and YO are inviable. Many workers have used this system. In some cases the markers used allowed first and second division errors to be distinguished while in others this distinction could not be made.

Bateman (1968) studied non-disjunction of chromosome 2 of *Drosophila* using isochromosome stocks. In these the two left arms (L) of the homologous pair were joined to one centromere (isochromosome 2L) and the two right arms (R) to another (isochromosome 2R). In females the isochromosomes disjoined regularly to give eggs with either 2L or 2R. In males segregation was random so that gametes with 2L, 2R, 2L + 2R or nullo-2 were found in approximately equal numbers. These latter two were disomic and nullisomic respectively. When isochromosome males were mated to normal females then all progeny resulting from normal disjunction in the female died because they were aneuploid for chromosome 2. If non-disjunction occurred in the female then both nullisomics fertilized by a disomic sperm and disomic eggs fertilized by a nullisomic sperm would have a balanced chromosome complement and would survive. As all progeny which received a normal chromosome complement from the female were inviable it was only possible to estimate the frequency of aneuploidy.
using egg counts to indicate how many normal eggs were produced.

Clark and Sobels (1973) studied non-disjunction of chromosome 2 using isochromosome 2 stocks of both males and females, the advantage being that both regular and exceptional progeny survived. Each isochromosome was marked to allow its identification in the progeny. Figure 1.2 illustrates the cross used and the possible progeny. It can be seen that for both regular and exceptional progeny, assuming random segregation in the male, only one quarter of the possible gametic combinations were viable, that is egg hatchability was expected to be twenty-five percent. Deviations from this would indicate non-random segregation in the male. The higher the probability of the right and left arms segregating together, the lower the egg hatchability but the greater the efficiency of recovering exceptional progeny. This system has the disadvantage that the stocks on which tests would be carried out have abnormal chromosomes. Isochromosomes, unlike normal metacentric chromosomes, have only a small region of homology at the centromere. Chromosome pairing at meiosis is therefore very limited in extent and it could be that the response to inducers would be different from that of normal chromosomes.

All Drosophila testing systems have the disadvantage that male meiosis in Drosophila is known to be different from man in that no recombination occurs. Female meiosis may also be different. Under certain experimental
Figure 12. Punnett square showing the viable (light) and non-viable (dark) zygotes obtained by crossing the two isochromosome stocks, \( j\); \( px\) females and \( b\); \( px\); \( vg\) males. Left isochromosomes are represented by full lines; right isochromosomes by broken lines. (from Clark and Sobels, 1973).
conditions it is possible to demonstrate that non-homologous chromosomes may disjoin regularly, as though they had been paired (the distributive pairing hypothesis of Grell, 1962). Whether this disjunction of non-homologous chromosomes occurs in man is unclear. Also in the female the chromosomes at meiosis are held in a chromocentre (Dävrine and Sunner, 1973). It may be that non-disjunction in *Drosophila* can occur through mechanisms not possible in man. However, non-disjunction in *Drosophila* also occurs in a more widely recognised way, that is failure of disjunction despite successful exchange (Merriam and Frost, 1964).

The usual advantages of low cost and convenience apply to *Drosophila*. In addition, it is capable of carrying out the same metabolic reactions as the mammalian liver (Sobels and Vogel, 1976). This would mean that any agents which affected non-disjunction indirectly, by being first metabolised into an active product might be detected in *Drosophila*. Such compounds are well known in mutagenic studies but no such aneuploid inducer has been described.

c) **Fungal Testing Systems**

There are two groups of fungal test systems, those which can detect mitotic aneuploidy and those which detect meiotic aneuploidy.

**Mitotic Systems**

Several systems have been developed to detect mitotic aneuploidy in diploid *Aspergillus nidulans* and these are illustrated in Figure 1.3. The principle of
the detection method is the same in all cases. Käfer (1961) showed that aneuploidy occurred frequently in diploid Aspergillus (1 - 2%) and that most detected aneu- ploids were hyperploids with 2n + 1 chromosomes. These were all unstable and stability was achieved through chromosome loss to give diploidy. If the original di- ploid was heterozygous then one third of the stable di- ploids resulting from such chromosome loss would be homo- zygous for both arms of the chromosome. Mitotic non- disjunction can be distinguished from mitotic crossing- over as the latter event generates homozygosity for genetic markers on one chromosome arm only. The system of Kappas et al. (1974) is therefore not ideal for aneuploid screening as chromosomes are only marked on one arm.

In the systems illustrated in Figure 1.3 methods of selection for homozygosity vary. In some cases conidia are plated on selective medium so only homozygous colonies will grow (Bignami et al., 1974; de Bertoldi et al. 1980). In other cases colonies are examined for the appearance of different coloured sectors, which indicate homozygosity has arisen (Kappas et al., 1974; Harsanyi et al., 1977; Normansell et al., 1979; Morpurgo et al., 1979). Morphologically abnormal colonies can also be selected as possible aneuploids (Harsanyi et al., 1977; Assinder and Upshall, 1982). In all cases the genotypes of the selected colonies are tested to differentiate mitotic recombination and aneuploidy.

The system of Bignami et al. (1974) has the
FIGURE 1. Mitotic aneuploidy detection systems in *Aspergillus nidulans*.

Kappas *et al.* (1974)

\[
\begin{align*}
\text{I} & : \quad \text{adF9} + yA2 + \quad \text{wa1} + \\
\text{II} & : \quad + + b1A1 + + \text{meth A17}
\end{align*}
\]

Homozygotes detected as white or yellow sectors
Aneuploidy and mitotic recombination cannot be distinguished.

Bignami *et al.* (1974); Morpurgo *et al.* (1979)

\[
\begin{align*}
\text{I} & : \quad + + \text{fpaAl anA1} + + \text{pabaA1} yA2 + + \\
\text{II} & : \quad + + \text{rifOAl} + + \text{proA1} + + \text{adE20 b1A1}
\end{align*}
\]

Homozygotes detected as pFPA resistant colonies
Aneuploidy confirmed by yellow conidia
Homologous abnormal sectors also examined for aneuploidy
Aneuploidy confirmed by pFPA resistance

Harsanyi *et al.* (1977)

\[
\begin{align*}
\text{I} & : \quad \text{su adE20 pabaA yA2 adE20} + + \\
\text{II} & : \quad + + + + \text{adE20} wA2 + \text{cmxE16}
\end{align*}
\]

Homologously abnormal sectors also examined for aneuploidy
Aneuploidy confirmed by whole chromosome type segregation of markers.

Normansell *et al.* (1979)

\[
\begin{align*}
\text{I} & : \quad \text{fpaB37 gal D5 suA1ad20} + \text{rifOAl anA1} \quad \text{pabaA1 yA2 adE20 b1A1} + + \\
\text{II} & : \quad + + + + \text{suA1} + + + + \text{adE20} +
\end{align*}
\]

Aneuploidy confirmed by pFPA resistance and expression of recessive markers on the left arm or by yellow colour.

de Bertoldi *et al.* (1980)

\[
\begin{align*}
\text{I} & : \quad \text{pim B10 proA1 pabaA1 yA2} + + + + b1A1
\end{align*}
\]

Aneuploidy confirmed by yellow conidia.

Assinder and Upshall (1982)

\[
\begin{align*}
\text{I} & : \quad + \text{proA1} + yA2 + + \quad + + \text{phen A2} \\
\text{II} & : \quad + + \text{adE20} + \text{actA1} + + \\
\text{IV} & : \quad + + \text{pyroA4} + + \text{facA303}
\end{align*}
\]

Morphologically abnormal colonies tested for aneuploidy.
Aneuploidy confirmed by whole chromosome type segregation of markers.
LEGEND TO FIGURE 1.3.

Linkage maps are not to scale. Only chromosomes used to study aneuploidy are shown. Other chromosomes were usually heterozygous for markers so as to establish that the colonies were not haploid. Gene symbols for auxotrophic markers are adE20, adF9; adenine; anAl, aneurin biAl, biotin; cnxE16, nitrite; galD5, galactose utilization methA17, methGl, methionine; nicA2, nicotinamide; pabaAl paraminobenzoic acid; phenA2, phenylalanine; proAl, proline; pyroA4, pyridoxine; riboAl, riboflavin; suadE20, suppressor of adE20. Resistance markers are acr, acriflavine; actAl, actidione; fac, fluoroacetate; fpaAl, fpaB37, p-fluorophenylalanine; pim, pimaricin; sulAl sulphanilamide. Conidial colour markers are wAl, wA2, white conidia; yA2 yellow conidia. Solid circles represent centromeres.
disadvantage that the test relies on the presence of pFPA in the medium. This is known to be a very potent aneuploid inducer and although pFPA is also present in the control platings there is always the possibility of a synergistic effect between pFPA and the test compounds. Morpurgo et al. (1979) avoided this difficulty by selecting different coloured sectors and then testing for pFPA resistance.

Parry and Zimmerman (1976) and Parry et al. (1979a) proposed the use of a yeast strain (D6) for detecting mitotic aneuploidy, the genotype of which is illustrated in Figure 1.4. The system detects chromosome loss from diploids by screening for cycloheximide resistant colonies. The strain is homozygous for an ade-2 mutation and colonies are therefore red. Cycloheximide resistant colonies arising from loss of chromosome VII are white because they are now expressing the recessive ade-3 mutation, which prevents the accumulation of red pigment. Initial experiments confirmed that the majority of white, cycloheximide resistant colonies expressed all markers on chromosome VII. As the markers are on both arms of the chromosome mitotic recombination is unlikely to have generated these colonies. This system only detects monosomy. On non-selective medium non-disjunction should produce colonies with white monosomic sectors and red trisomic sectors. White sectors were tested for multiple nutrient requirements. If these proved to be monosomic the red sector was tested for trisomy. The colonies were streaked out on sporulation medium. Five per cent produced four spored asci of which
FIGURE 1.4 Mitotic aneuploid detection systems in *Saccharomyces cerevisiae*.

Chromosome VII

```
ade-3 - leu-1 trp-5 cyh-2 met-3

+ ade-6 + + + +
```

Chromosome XV

```
ade 2-40
```

Monosomics detected as white cycloheximide resistant colonies. A homozygous ade 2-40 genotype confers red colony colour unless ade-3 is expressed.

Gene symbols for auxotrophic mutants are *met*, methionine; *trp*, tryptophan; *leu*, leucine and *ade*, adenine. *cyh* represents cycloheximide resistance. Solid circles represent centromeres. The linkage map is not to scale. (Parry and Zimmerman, 1976).
ten per cent (15) gave complete germination. Of these asci fourteen showed segregation of markers consistent with the original red sector having been trisomic. This analysis showed that at least a fraction of half-sectored colonies had monosomic and trisomic cells. However, it was not a suitable method to test for aneuploid induction as the proportion of sectored colonies which are aneuploid is uncertain and plating on non-selective medium would require large numbers of platings at a low density to get sufficient results. This strain is therefore only useful for examining the induction of monosomy.

A major objection applies to this and all other mitotic testing systems. Although mitotic non-disjunction may be an important indicator that agents do affect meiotic non-disjunction, there is a range of meiotic defects which cannot be examined in a mitotic system. The first division of meiosis is very different from a mitotic division and there are indications that it is, at least partially, under separate genetic control. This is suggested by the findings that along with mutants affecting both meiotic divisions there are meiotic mutants which affect only the first meiotic division and others which are specific to the second division and subsequent mitoses. It is therefore possible to interfere with one of the meiotic divisions but not the other. In Drosophila there is a testis specific β tubulin gene, which when mutant causes abnormal meioses, and non-disjunction is observed (Kemphues et al., 1980). Since the tubulin is testis specific it is possible that
it is a meiotic spindle component. If the tubulin in meiotic cells is different from that in mitotic cells it may be that meiotic divisions are vulnerable to attack by different chemical agents than mitotic divisions. Also, as errors at the first meiotic division are one of the major causes of aneuploidy in man (Jacobs and Morton, 1977), screening methods which specifically exclude examination of this division are not as useful as those which allow effects of agents on this division to be monitored.

**Meiotic Systems**

The fungal meiotic testing systems include those using *Saccharomyces cerevisiae*, *Neurospora crassa* and *Sordaria brevicollis*. The systems are of two types, those based on random spore analysis and those based on ascus analysis.

**Random spore plating**

There are two random spore plating systems in *Neurospora* and two in yeast. Parry (1977) and Parry et al. (1979a) proposed a system in yeast using strain D9J2, the genotype of which is illustrated in Figure 1.5. D9J2 carries a series of recessive markers in repulsion on both arms of chromosome VII and is also heterozygous for uracil requirement and canavanine resistance. First division errors will produce disomic products which can grow on minimal medium plus uracil. These can therefore be selected by plating on this medium plus canavanine. The presence of canavanine excludes growth of any diploids which have not gone through meiosis. Only fifty percent of disomic
FIGURE 1.5 Random spore plating methods to detect meiotic aneuploidy in *Saccharomyces cerevisiae*

a) Parry *et al* (1979a)

\[
\begin{align*}
\text{VII} & : \text{ade-3} + \text{leu-1} + \text{cyh-2} + \text{aro-2} + \text{ade-5} \\
\text{V} & : \text{can-1} \\
\text{XL} & : \text{ura-1}
\end{align*}
\]

Plated on minimal medium plus canavanine + uracil

Diploids: No growth on plating medium

Haploids: No growth on plating medium

Disomics arising at 1st \( \frac{1}{2} \): 50% grow on plating medium if no crossing-over between markers

Disomics arising at 2nd \( \frac{1}{2} \): Only detected if crossing-over between *leu-1* and centromere and *ade* loci and centromere.

b) Sora *et al* (1982)

\[
\begin{align*}
\text{V} & : \text{can-1} + \text{his-1} + \text{ilv-1} + \text{met-5} \\
\text{VII} & : \text{cyh-2} \text{ leu-1} \text{ ade-2} \\
\text{XL} & : \text{ura-1}
\end{align*}
\]

Plated on minimal medium plus adenine, leucine, uracil and cycloheximide

Diploids: detected as sporulating colonies

Haploids: cannot grow on plating medium

Disomics arising at 1st \( \frac{1}{3} \): 1/3 detected; 2/3 lost as either cycloheximide sensitive and/or auxotrophic due to crossing-over in *hom*-centromere interval. Crossing-over in other intervals ignored.

Disomics arising at 2nd \( \frac{1}{3} \): 1/3 detected; 2/3 lost as either cycloheximide sensitive or auxotrophic due to no crossing-over in the *hom*-centromere interval.

The frequency of first division errors (p) and second division errors (q) determined by replica plating on uracil. \( p + q = 1 \) and frequency of uracil requirers = \( p(0.04) + q(0.42) \).
Legend to Figure 1.5

Solid circles represent centromeres. The linkage maps are not drawn to scale. Numerals below chromosomes represent map distances. Roman numerals indicate chromosome numbers. Only those markers involved in the aneuplod detection system are shown. Other chromosomes were generally marked with heterozygous markers. Gene symbols for auxotrophic markers are: \textit{ade}, adenine; \textit{arg}, arginine; \textit{aro}, aromatic biosynthesis; \textit{his}, histidine; \textit{hom}, homoserine; \textit{ilv}, isoleucine or valine, \textit{leu}, leucine, \textit{lys}, lysine; \textit{met}, methionine and \textit{trp}, tryptophan. Resistance markers are \textit{can}, canavanine and \textit{cyh}, cycloheximide.
colonies will grow, that is those which inherit the canavanine resistance mutation. The proportion of first division errors detected will also be reduced by crossing-over in the marked interval of chromosome VII. If crossing-over occurs in this interval and a first division error arises, fifty percent of the resulting disomics will be homozygous for some of the auxotrophic markers. Second division errors will not be detected unless crossing-over occurs between ade-6 and the centromere and leu-2 and the centromere. Otherwise all disomics arising through second division errors will be homozygous for at least one auxotrophic marker. This system should allow rapid detection of chemicals inducing first division non-disjunction. However, there are some unstated technical difficulties preventing its use in routine screening (Parry et al., 1979a).

A second random spore plating method using yeast has recently been described which has the additional advantage of being able to detect aneuploidy arising from second division errors and diploidy arising from failure to reduce the chromosome number (Sora et al., 1982). This system is based on selecting colonies heterozygous for markers on the right arm of chromosome V. The genotype of the strain used is illustrated in Figure 1.5. This also shows the proportions of disomics, arising through first and second division segregation errors, which are viable on selective medium containing adenine, leucine uracil and cycloheximide. Second division errors can be
detected because markers used for selection are all on
the same chromosome arm. Crossing-over in the centromere-
hom interval allows detection of second division errors.
Replica plating on medium minus uracil gives the frequency
of uracil requirers. From this it is possible to calculate
the proportions of first and second division errors detected,
knowing the map distance of the ura-3-centromere interval.

Diploids are detected as growing colonies which spor-
ulate when placed on sporulation medium. Only a/α diploids
will be detected in this way. a/a and α/α diploids will
not sporulate. The diploids cannot result from cells
which did not go through meiosis as these would not survive
on cycloheximide. They must therefore be the products of
an aberrant meiosis such as endomitosis or failure of
either meiotic division or due to random nuclear fusion
within a tetrad, after meiosis. These different hypotheses
lead to different expectations for the segregation of the
leu and ade markers on chromosomes VII and XV respectively.

This system is therefore capable of detecting first
and second division errors as well as diploidy arising
through aberrant meioses. Discriminating these errors
involves several extra platings following growth on selective
medium. A random spore plating method using Neurospora
crassa has been described which uses parental strains
giving a cross heterozygous for auxotrophic markers on
linkage group 1 (Griffiths and Delange, 1977; Griffiths,
1979). The strains are illustrated in Figure 1.6.
Disomy for linkage group 1 is detected by plating on
FIGURE 1.6 Random spore plating methods to detect meiotic aneuploidy in *Neurospora crassa*

a) Griffiths and Delange (1977)

\[
\begin{array}{cccccccc}
\text{leu-3} & + & \text{a} & \text{arg-1} & + & \text{ad-3A} & + & + & \text{al-1} \\
\text{un-3} & A & \text{QII} & \text{9} & + & \text{al-2} & + & 28 & <1 \\
\text{10} & 41 & 4 & 28 & <1 \\
\end{array}
\]

Plated on medium lacking adenine, arginine and nicotinamide

Diploids/Heterokaryons: 7/8 formation prevented by heterokaryon incompatibility factors C/c D/d E/e

Haploids: cannot grow on plating medium

Disomics arising at 1st: grow on plating medium, tol overcomes A/a incompatibility

Disomics arising at 2nd: cannot grow unless crossing-over occurs between ad-3 and the centromere (also in some cases arg-1 and centromere)

leu-3 and un-3 were redundant markers. C/c, D/d and E/e also prevent multiple disomy involving chromosomes II and VII

b) Smith and Yorston (1981)

\[
\begin{array}{ccccccc}
\text{pyr-3} & \text{his-5} & + & + \\
\text{his-5} & \text{leu-2} \\
\end{array}
\]

Plated on minimal medium

Diploids/Heterokaryons: can grow on minimal medium

Haploids: cannot grow on minimal medium

Disomics arising at 1st: grow on minimal medium unless crossing-over on the pyr-3-centromere interval.

Disomics arising at 2nd: grow on minimal medium if crossing-over in pyr-3 interval.
Legend to Figure 1.6

Solid circles represent centromeres. Linkage maps are not drawn to scale. Only markers involved in aneuploid detection are shown. Auxotrophic markers are \textit{arg}, arginine; \textit{his}, histidine, \textit{leu}, leucine; \textit{nic}, nicotinamide, \textit{pyr}, pyrimidine. \textit{al} represents albino-spores and \textit{un} unknown heat sensitive defect. Roman numerals indicate chromosome numbers. Arabic numerals below chromosomes indicate map distances.
selective medium lacking arginine, adenine and nicotinamide. Any disomic arising through first division errors should be able to grow on such medium unless crossing-over has occurred between the markers. The **arg-1**, **ad-3A**, **ad-3B** and **nic-2** markers were chosen because they are tight mutations and strains are interfertile. Selection is imposed against heterokaryotic (as opposed to aneuploid) colonies by incorporating heterokaryon incompatibility loci into the strains. Only one-eighth of possible heterokaryons will be able to grow as there are three such loci included. Triple recombination to produce a prototroph for arginine, adenine and nicotinamide would be expected at a frequency of $10^{-6}$ which is less than the frequency of prototrophic colonies observed (mean = $4.9 \times 10^{-5}$). Detailed analysis of these colonies showed them to be disomic and non-recombinant.

This system will not detect second division errors as disomic products arising in this way will be homozygous for two of the selection markers. The advantage of this system over that of Parry *et al.* (1979) is that the tight linkage of the selection markers reduces the probability of aneuploids going undetected due to recombination between the markers. A disadvantage of the system is that in order to be sure that no heterokaryons are forming microscopic examination of prototrophic colonies is necessary. This has to be carried out two days after plating to check that growth has originated from a single spore. This will reduce the efficiency of a random spore plating method.
considerably. Unlike the yeast system of Sora et al. (1982) there is some variation in control levels of aneu-
plody. This problem has not been discussed for the other fungal meiotic test systems.

Smith and Yorston (1981) have published a brief account of a second random spore plating method using N. crassa. This uses complementary alleles of the his-5 locus on linkage group 4. Parental strains are illustrated in Figure 1.6. Disomic colonies arising through first division errors and recombinants between the his-5 alleles will grow on minimal medium. Fifty percent of first division errors following recombination between the centromere and any marker will go undetected. Second division non-disjunction will be detected if there is recombination in the pyr-3-centromere interval. In order to determine whether prototrophs detected using this system are aneu-
ploid or recombinant it is necessary to cross them on to other strains. Disomic spores will behave as hetero-
karyons, heterozygous for leu-2 and pyr-3, while recom-
binants should be homokaryotic for these loci. Perhaps once the system is fully characterised and the proportion of prototrophic colonies due to recombination between the his-5 alleles is determined, then this further analysis would not be necessary. However, no consideration is given to the problem of heterokaryon formation in this system. Unless the probability of this event is reduced as much as possible it would still be necessary to subject growing colonies to further analysis. This would make the system inefficient.
Ascus analysis

Threlkeld and Stoltz (1970) reported strains of *N. crassa* suitable for analysis of non-disjunction. The B3 and B5 alleles at the pan-2 locus on linkage group VI are complementing mutants and when present in the same nucleus produce spores with black, wild type colour. On medium with reduced levels of pantothenate, spores with either allele present in the nucleus are pale in colour. In crosses of pan-2(B3) x pan-2(B5) on such medium asci containing equal numbers of black and abortive spores were detected. On dissecting these asci the black spores were found to be disomic for linkage group VI. There was no evidence for disomy of linkage group I or for heterokaryosis. Various asci containing black disomic spores were found which were classified into four different types:

Class I : asci with four black and four abortive spores segregating at the first meiotic division.

Class II : asci with four black and four abortive spores in a second division type segregation.

Class III : asci with two black, four pale and two abortive spores, the black and abortive spores always being in opposite halves of the ascus.

Class IV : asci with two black, two abortive and four pale spores, the black and abortive spores always being in the same half of the ascus.

Class I asci are assumed to arise through non-disjunction or non-conjunction at the first meiotic division and Class III asci are consistent with premature centromere division having occurred at the first meiotic division.
The analysis of ordered tetrads gives a clear indication that such an error can occur. Class IV asci could arise through second division non-disjunction or premature centromere division at the second meiotic division, following crossing-over between pan-2 and the centromere. Asci in Class II could be due to a double event from Class III or Class IV. However, the frequencies of the different classes suggested that the majority of Class II asci are due to two cases of premature centromere division at the first meiotic division. Analysis of black spores revealed no cases of a stable pseudowild type. In all cases heterokaryotic cultures were obtained, often with three or more nuclear types. This suggested that before the disomic nucleus regains the haploid number it is readily involved in mitotic recombination.

A second ascus analysis method for detecting meiotic aneuploidy has been described by Bond (1976), Bond and McMillan (1979a,b), Fulton and Bond (1983). This is based on a similar principle to that of Threlkeld and Stoltz, but uses the ascomycete fungus Sordaria brevicollis, which has a life cycle similar to Neurospora. The Sordaria system uses two complementing spore colour mutants, at the buff (bl) locus on linkage group II, called S6 and C70. When present in the same nucleus these form black, wild type ascospores which are easily distinguishable from the background of buff spores. Aneuploids are detected by examining asci under a compound microscope. Disomic spores will be black and, if the result of non-disjunction,
non-conjunction or premature centromere division will be
accompanied by an equal number of nullisomic spores.
Black spores are usually found with abortive spores which
are assumed to be nullisomic. Dissection of asci and
germination of black spores has shown them to be pseudo-
wild types which break down when cultured to give an S6/C70
heterokaryon. That the black spores are not diploid has
been illustrated by the fact that the cultures are always
homokaryotic for mating type. It has been demonstrated
that the spores are not multiply disomic for linkage group
I and II but this has not been shown for the other linkage
groups.

As in the case of Threlkeld and Stoltz (1970) the
examination of asci can yield information about the
meiotic error giving rise to aneuploidy. Asci with four
black and four abortive spores segregating at the first
meiotic division are consistent with non-disjunction or
non-conjunction at the first meiotic division. Asci with
an alternate 2:2:2:2 sequence of black and abortive spores
are also found. Threlkeld and Stoltz attributed such
asci to a double premature centromere division at the first
meiotic division. However, in Sordaria, another explanation
is possible. Sordaria and Neurospora are very different in
the frequency of spindle overlap. This is also known as
nuclear passing and results in the alteration of the spore
sequence within an ascus. Complete spindle overlap at the
second meiotic division causes one set of daughter nuclei
to be separated by the other set so that, for example, a
4:4 sequence becomes 2:4:2. Berg (1966) showed that complete spindle overlap at the second meiotic division was rare (<0.1%), and its effects on ascus type frequencies minimal. Partial spindle overlap at the second meiotic division will switch the position of the middle spore pairs so that a 4:4 sequence will become 2:2:2:2 and vice versa. Estimates of its frequency using genetical methods are complicated by the fact that the frequency of overlap varies depending on the centromere distance of the marker used to detect it (Whitehouse, 1957) and also by the possibility that partial spindle overlap asci may be preferentially discharged. The frequency of spindle overlap would then fall as the cross matures (MacDonald, 1976). However, Mu'Azu (1973) estimated the frequency cytologically and found that in approximately fifty percent of asci the second division spindles showed partial overlap. As asci with four black and four abortive spores arise from a first division segregation error this is essentially the equivalent of a centromere linked marker. The frequency of 2:2:2:2 asci is therefore not affected by centromere distance. The high frequency of spindle overlap normally found in *Sordaria* crosses means that there is no need to postulate any origin other than first division non-disjunction or non-conjunction to account for the almost equal frequencies of 4 black : 4 abortive and 2 black : 2 abortive : 2 black : 2 abortive asci.

Bond (1976) described how asci with two black, two abortive and 4 buff spores were consistent with second
division non-disjunction following a cross-over between the buff locus and the centromere. He also detected asci with black but no abortive spores. A full description of the various ascus types and their origins will be found in Chapter 3 of this thesis. It is sufficient here to point out that different ascus types can be found, and as with the Neurospora system of Threlkeld and Stoltz (1970), the meiotic errors giving rise to such asci can be inferred.

It is clear from the above descriptions that an ascus analysis system has the advantage over a random spore plating method in that the types of aneuploidy occurring can be identified. The disadvantage is that scoring of aneuploidy is more laborious, as microscopic examination of large numbers of asci is required.

A system which could detect aneuploidy for a linkage group by either ascus analysis or random spore plating would be more flexible. According to how much, or what, information was wanted about induction, either system could be used.

It was mentioned earlier that a major problem when using lower eukaryotic systems is extrapolation to man. In order to assess their usefulness, it would be necessary to test agents known to induce aneuploidy in mammals, to see if these proved positive in the lower eukaryotic systems. It may be that lower eukaryotic systems detect induction of distinguishing some errors but not others. The possibility of the types of aneuploidy being induced would allow this question to be answered.
d) **Summary**

The problem therefore is that the high incidence of aneuploidy in man has raised the possibility that he may be at risk to environmental agents inducing even higher levels. Aneuploidy can arise through a wide range of meiotic errors and many different classes of inducer could exist, attacking at different points. Any testing system should therefore be able to check for induction of as many classes of aneuploidy as possible. Although a mammalian system would be the most suitable to avoid many problems of extrapolation, these have to be excluded as an initial mass screening method due to the time and high expenditure necessary to run them. *Drosophila* systems would be financially more acceptable but it is known that meiosis, at least in the male, and possibly in the female, is different from that in man. Some *Drosophila* testing systems are based on stocks constructed with highly abnormal chromosomes in order to allow identification of aneuploid progeny. Whether it is justified to use such abnormal situations to consider what may occur in other animals is questionable. Fungal systems which investigate mitotic aneuploidy exclude a major class of possible errors from analysis. Meiotic fungal systems may be useful as the stocks are inexpensive to maintain and the actual tests are relatively rapid. The major problem is with extrapolation to man, for example fungal meiosis may also be atypical as unlike higher eukaryotes the nuclear membrane remains intact during the division (Fincham et al., 1979).
No single lower eukaryotic system is likely to be wholly satisfactory. The most sensible course would seem to be to fully characterise potential aneuploid testing systems so that spontaneous levels are known, and to investigate their response to known aneuploid inducers, especially those which affect a component of meiosis thought to be different in lower and higher eukaryotes.

6. **The Aims of this Project**

The aim of this project was to develop the *Sordaria brevicollis* system to be a useful aneuploid testing system, both to use as a screening method and also to ask questions about the mechanisms of aneuploid induction. To achieve this broad aim subsidiary objectives were:

a) To characterise the **buff** (linkage group II) system described by Bond (1976) so that the degree of fluctuation in controls was known and to determine the spectrum of aneuploid types seen in the controls.

b) To follow up the observation by Dr H.L.K. Whitehouse that crosses of mutants at the **grey-6** locus on linkage group IV produced asci with four black and four abortive spores, at low frequency. There was a possibility that this could provide a system for detecting aneuploidy, on a similar basis to the buff system. It was decided to characterise the grey-6 system and compare spontaneous aneuploidy in linkage group II and linkage group IV.

c) Earlier work by Bond and McMillan (1979a,b) had shown the *Sordaria* buff detection system was sensitive to aneuploid induction by p-fluorophenylalanine (pFPA). A full analysis of aneuploid induction by pFPA, investigating the effects on all the ascus
types had not been carried out. It was decided to do this analysis and to compare the response to pFPA of linkage group II and linkage group IV.

d) The time necessary to score *Sordaria* crosses for induction of aneuploidy could be reduced by having a random spore plating method complementary to the ascus analysis system. This would require markers on either linkage group II or IV and it was hoped to obtain some markers from filtration enrichment or resistance mutant hunts.

e) Assessing the usefulness of a lower eukaryotic system requires testing it with a range of known aneuploid inducers. It was hoped to be able to carry out such tests on both random spore plating and ascus analysis methods. In a limited time period the number of possible inducers which could be tested would be restricted. It was decided to test spindle inhibitors and X-rays. The reason for this selection will be explained in the appropriate chapters.

f) In order to investigate the contributions of non-disjunction and non-conjunction to aneuploidy arising at the first meiotic division it is necessary to have markers loosely linked to the *buff* or *grey-6* locus. It was hoped to find such markers in filtration enrichment or resistance mutant hunts. Any possible loosely linked markers would be mapped by aneuploid mapping, as would the two already available in the laboratory, *met-6* and *lys*. 
CHAPTER 2: MATERIALS AND METHODS

This chapter outlines those procedures which were used throughout the project. Details of any more specific methods are presented in the appropriate chapter.

Strains used

All strains were isolated from the Sordaria brevicollis stocks of D.J. Bond, unless otherwise stated. The auxotrophic and spore colour mutants used are listed in table 2.1 and 2.2. The morphological mutant rhythmic growth (rg) was also used. This grows with very frequent hyphal branching, and occasional escapes, which have low frequency branching, followed by more high frequency branching. Concentric rings of growth are formed in this way, and a more colonial growth than normal is observed. There are two components involved in rhythmic growth, one linked to mating type, the other unmapped.

Strains were reisolated from stock crosses, by ascus dissection or by spreading and picking spores. This was necessary as cultures gradually lost fertility and so had to be replaced.

The buff and grey isolates used in aneuploidy testing were therefore newly isolated for each set of experiments. As isolates of each mutant were always obtained from the same stock cross, or a cross of daughter spores, they were not known to have any
<table>
<thead>
<tr>
<th>Linkage group</th>
<th>Marker</th>
<th>Locus</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>methionine</td>
<td>met-4</td>
<td>Complementation tests</td>
</tr>
<tr>
<td>II</td>
<td>nicotinamide or tryptophan methionine</td>
<td>not-1 not-2 met-1</td>
<td>Possible markers for random spore plating Markers in aneuploid testing crosses</td>
</tr>
<tr>
<td>III</td>
<td>none available</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>* methionine RD6 methionine SF1 uracil * pyridoxine * riboflavin</td>
<td>met-2? ura pdx ribo</td>
<td>(complementation tests (aneuploid testing (random spore plating) random spore plating (considered for use in random spore plating)</td>
</tr>
<tr>
<td>V</td>
<td>methionine</td>
<td>met-5</td>
<td>complementation tests</td>
</tr>
<tr>
<td>VI</td>
<td>methionine acetate</td>
<td>met-3 ace</td>
<td>complementation tests isolated in mutant hunt</td>
</tr>
<tr>
<td>VII</td>
<td>methionine</td>
<td>met-7</td>
<td>complementation tests</td>
</tr>
<tr>
<td>?</td>
<td>methionine</td>
<td>met-6</td>
<td>complementation tests aneuploid mapping</td>
</tr>
<tr>
<td>?</td>
<td>lysine</td>
<td>lys</td>
<td>aneuploid mapping</td>
</tr>
</tbody>
</table>

* Gift of Dr. H.L.K. Whitehouse
### TABLE 2.2 Spore colour mutants used

<table>
<thead>
<tr>
<th>Linkage group</th>
<th>Spore colour mutant</th>
<th>Locus</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>C33</td>
<td>No name</td>
<td>Mapping</td>
</tr>
<tr>
<td>II</td>
<td>buff S6</td>
<td>buff-1</td>
<td>Mapping, aneuploid testing system</td>
</tr>
<tr>
<td></td>
<td>buff C70</td>
<td>buff-1</td>
<td>Mapping</td>
</tr>
<tr>
<td></td>
<td>yellow</td>
<td>ylo-9</td>
<td>Mapping</td>
</tr>
<tr>
<td>III</td>
<td>C12</td>
<td>gray-2</td>
<td>Mapping</td>
</tr>
<tr>
<td>IV</td>
<td>*grey YS18</td>
<td>grey-6</td>
<td>Mapping, aneuploid testing system</td>
</tr>
<tr>
<td></td>
<td>grey B9</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>grey RW25</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>grey C31</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>grey YS121</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>S229</td>
<td>ylo-4</td>
<td>Mapping</td>
</tr>
<tr>
<td>VI</td>
<td>hyaline</td>
<td>hyaline</td>
<td>Mapping</td>
</tr>
<tr>
<td>VII</td>
<td>C20</td>
<td>grey-7</td>
<td>Mapping</td>
</tr>
</tbody>
</table>

*All grey-6 mutants were the gift of Dr. H.L.K. Whitehouse*
major genetic differences, but the possibility that they may have done should be borne in mind.

Media

All medium recipes can be found in Appendix I.

Crossing techniques

Crosses were carried out on corn meal agar, and incubated at 25°C. Routinely there was 20 ml CMA per 90 mm diameter plastic Petri dish. Two methods of crossing were used.

1) Confrontation crosses. The isolates to be crossed were inoculated at opposite sides of the Petri dish, about 10 mm from the edge of the plate. Occasionally two inocula of each isolate were used and placed alternately round the plate at regular intervals. This yielded up to twice the material of the first arrangement. The crosses were incubated for 10 days, when mature perithecia were found along the line where the colonies met. This method was a rapid and economic way to set up crosses and was used routinely for stock crosses. However, it had the disadvantage that, as the colonies met in an arc, not all perithecia were initiated simultaneously, and so were of mixed maturity. Also, the yield from such crosses was not very large.

2) Fertilization crosses. The isolates to be crossed were inoculated onto separate plates. One isolate was designated female, the other male and plates were set up in the ratio 3 female:1 male, and incubated for four
days. After this time sterile, distilled water was poured over each male plate and the surface rubbed with a sterile glass rod, to form a microconidial suspension. This was distributed over three female plates which were lightly shaken to ensure even fertilization. Excess water was poured off and the plates incubated for a further six days. Although more time consuming than confrontation crosses, this method had the advantage of simultaneous fertilization of protoperithecia and so maximised the number of mature perithecia obtained on harvesting. Yield could be further improved by placing sterile cellophane membranes on the surface of female plates, prior to inoculation. The fungus then grew on the surface of the membrane. The fertilization method plus membranes was used routinely for aneuploid testing unless otherwise stated. Setting up crosses on membranes also meant that crosses could be easily transferred to other medium if required.

**Harvesting crosses and storing material**

Mature crosses were harvested by scraping perithecia off the plates using a tungsten needle or the edge of a glass coverslip. The perithecia were placed in a drop of 10% (w/v) sucrose solution on a slide. They were crushed open under a coverslip, which was then removed and the slide left to dry. The material could then be stored until required. Material for up to four slides could be harvested from a good cross on membranes.
Resuspension in 10% sucrose solution allowed retrieval of material for reisolation or examination.

**Scoring crosses for aneuploidy**

Following resuspension in 10% sucrose, rosettes of asci were transferred to a block of 4% agar, flooded with sucrose solution. This was left until almost dry, and a coverslip placed over the material. To avoid bias in picking rosettes, transfer was carried out at X 16 magnification, at which individual asci could not be distinguished. The sucrose prevented uneven drying out of the agar, the agar block facilitated removal of the coverslip, if required. A completed slide had 40-50 rosettes and was examined systematically at X 100 magnification. The number of asci in each rosette was estimated to the nearest ten and recorded. The spore sequence in asci containing aneuploid spores was checked at X 400 magnification. If it was still unclear the position of the rosette was marked. When the remainder of the slide was scored the coverslip was removed and the rosette containing the problem ascus cut out on agar, and transferred to a drop of sucrose solution on a slide. The ascus was teased out of the clump and the spore sequence determined. Occasionally this was unsuccessful and the ascus had to be recorded as unclassified.

For any single treatment slides were scored until fifty aneuploid-containing asci had been observed. A
fixed number was chosen to avoid introducing a biassing factor when choosing to stop counting. Fifty was an arbitrary number which would hopefully reduce any random 'jackpot' effect while allowing counts to be obtained in a reasonable time. To count fifty such asci in a typical control cross would take approximately two to three working days, counting full time.

The estimation method of obtaining the total number of asci was assumed reasonably accurate, as repeat counts of the same material, or material from the same cross, gave similar results. Bond (1976) recorded results in which the frequencies obtained using this estimation method were compared to frequencies of aneuploidy obtained after teasing rosettes apart. There was very good agreement between the frequencies obtained using the two methods.

Mating type tests

Wild type isolates of each mating type, A and a, were inoculated in the centre of separate plates of CMA. These were incubated for 4 days at 25°C, or 3 days at 29°C. A small piece of the isolate of unknown mating type was then scraped over the surface of each plate, and these were incubated overnight. The following day the plates were examined for signs of perithecial formation. This only occurred on the plates of opposite mating type to the isolate. Ten to twelve isolates could be tested per plate.
Random spore isolation and ascus dissection

Spreading random spores was a rapid method to obtain fresh isolates. A rosette of asci was placed in a drop of sterile water, on a plate of dissecting agar. A supplement of 0.7% w/v of sodium acetate was added to the dissecting agar, except when buff isolates were required. These do not require acetate to stimulate germination. The rosette was teased apart and the spores spread over the plate, using a sterile glass spreader. The plate was left overnight at room temperature and then germinated spores picked into tubes containing 1 ml of appropriately supplemented Vogel's (1956) N medium.

Ascus dissection was carried out on a block of 4% agar at a magnification of X 40. Spores were then picked into tubes, as above.

Auxotrophy tests

Growth requirements of isolates were tested by transferring small amounts of a culture into tubes of variously supplemented Vogel's (1956) N medium and examining tubes after 3 days incubation at 25°C. The transfer of mycelium was achieved in one of two ways. a) When a small number of tests was required mycelium was transferred using a tungsten needle, b) When there was a large number of tests required mycelium was transferred by suspension in water. Approximately 0.5 ml of sterile, distilled water was
added to each culture by Pasteur pipette. The surface of the culture was scraped gently with a pipette and the suspension sucked up. Two drops were dispensed to each test medium. Before using this method routinely tests were carried out to ascertain that results obtained were accurate and that wetted cultures were still suitable for subculture. Scraping the surface of the mycelium was necessary, as tests failed if the water was removed immediately after addition.

**Complementation tests with auxotrophs**

These were carried out on Vogel's (1956) N minimal medium in 50 mm diameter plastic Petri dishes. Strains of opposite mating type show vegetative incompatibility and so complementation tests are only successful using strains of the same mating type. A small inoculum of each isolate to be tested was placed in the centre of a plate. Each isolate was also inoculated on to a separate plate of minimal medium, as controls. Plates were examined after 2-3 days incubation at 25°C, growth on only the doubly inoculated plates indicating complementation.

**Filtration enrichment mutant hunt**

The principle of a filtration enrichment mutant hunt is to treat the fungus with a mutagen and then to provide conditions in which all but auxotrophic spores can grow. All growing colonies are filtered off, until hopefully only auxotrophic spores remain in the medium.
These are then plated in complete medium and tested for nutrient requirement. In the experiments reported here the strains used carried the rhythmic growth mutation. The colonial growth caused by this mutation facilitated filtering of the growing colonies. The procedure used was as follows.

1) The protoperithecial parent (female) was inoculated on to CMA in a conical flask, two inoculations per flask. 100 ml conical flasks were used containing 40 ml medium, slanted.

2) Two days later the microconidial parent (male) was inoculated on to CMA, in a Petri dish. One male parent was set up for every two females.

3) All procedures involved in UV irradiation and fertilization were carried out in a sterile room. The microconidial suspension was obtained from the male parent, four days after inoculation and filtered through muslin to remove any debris.

4) The suspension was placed in a crystallising dish and exposed to UV irradiation from a UV lamp (Hanovia 772/64) at a distance of 270 mm. Three times of exposure were used, 60s, 120s and 180s.

5) Females were fertilized with approximately 3 ml of suspension and kept in darkness for 24 hours to prevent photoreversion.

6) Crosses were left to mature for at least ten days.
7) Spores were harvested in sterile water.
8) The spore suspension from each cross was poured into galactose liquid minimal medium, 40 ml/250 ml conical flask. Tetracyclin was included in the medium to prevent bacterial contamination.
9) Flasks were shaken at 160-170 revs in an orbital incubator at 29°C. Growing colonies were removed by filtration through muslin. Filtration was carried out as often as growth dictated, that is as often as there were some visible colonies in the medium. Filtrations were stopped after no growth had been observed for 24-48 hr.
10) The remaining medium was added to 100 ml plating medium and distributed over five plates. Plating medium was more concentrated than normal galactose solid complete medium, to allow for the dilution effect due to addition of approximately 40 ml of liquid.
11) Growing colonies were picked into tubes of complete medium.
12) Cultures were tested on minimal medium. Any auxotrophs were tested for nutrient requirement, using multiply supplemented tubes in an adaptation of the method of Holliday (1956). Six tubes were supplemented with a combination of amino acids or vitamins such that requirement for any one nutrient gave growth in a unique combination of tubes.
Resistance mutant hunts

The principle of a resistance mutant hunt is to plate spores in complete medium containing a concentration of an antimetabolite, greater than or equal to the minimum inhibitory level. This is the minimum concentration which inhibits growth. The procedure is as for steps 1-7 of the filtration enrichment mutant hunt. Spores are then plated in complete medium containing the appropriate concentration of the selected compound and plates examined for growing colonies after 2-5 days incubation at 25°C. Growing colonies are picked and crossed to wild type to test for inheritance of resistance.

Statistical Procedures

The following statistical tests have been employed.

1. Replicated goodness of fit (G-statistic). This was the statistic used to test for heterogeneity among buff or grey-6 controls. The $G_H$ test ($G$ for heterogeneity) is a test of heterogeneity of data and $G_H$ values are compared against $\chi^2$ tables. The $G_H$ values are obtained from $f \ln f$ transformations of data as described by Sokal and Rohlf (1969).

2. Test of induction over control levels ($t'$ test)
   a) when only one induction level is available

   \[
   t = \frac{d}{\sigma_d} \quad \sigma_d = \sqrt{\frac{P_1}{N_1^2} + \frac{P_2}{N_2^2}}
   \]

   \[
   d = p_1 - p_2
   \]
P = no. of aneuploid containing asci
N = total number of asci counted in a particular treatment
p = the frequency of aneuploidy

This test assumes the results follow a Poisson distribution and that p is very small so that \((1 - p) = 1\).

b) When more than one induction experiment has been carried out a t test of paired differences is applied.

\[
t = \frac{\bar{d}}{\sigma/\sqrt{n}}
\]

\(d = \) difference between control and treated frequency

3. **Comparison of spectra (2 x 2 heterogeneity \(\chi^2\))**

This standard test was used to compare spectra of control and treated crosses.

4. **Comparison of ratios (2 x 2 heterogeneity \(\chi^2\))**

This standard test was occasionally used to compare ratios.

5. **Dose response of induction (linear regressions)**

Linear regressions were computed and tested for statistical significance, to determine whether there was any dose related response to chemical treatment.
The *Sordaria brevicollis* aneuploid detection system outlined in chapter 1 is based on spore colour mutants at the *buff* locus on linkage group II and will be referred to as the *buff* aneuploid detection system. Although the basic *buff* system has been described (Bond, 1976 and Bond and McMillan, 1979a,b) and the various types of asci with aneuploid spores classified (Bond, 1983) a full description of the system is not generally available (the latter publication being in press). A full account is therefore given of the ascus types which can be detected. It had been reported from work on the *buff* system that there was no variation in spontaneous aneuploid frequencies between replicate crosses in a single experiment but that variation did exist between control levels of aneuploidy in different experiments (Bond and McMillan, 1979). In *Drosophila*, when carrying out a sex-linked lethal test it is common practice to use a historical control, because over many years it has been established that the frequency of sex-linked lethals is normally no more than 0.5% (Kilbey et al., 1981). Only periodic checks are made on control levels, with a resultant large saving in time and effort. If the variation in *Sordaria* crosses could be more clearly understood it might be possible to use historical controls in a similar way and this would markedly reduce the time and labour necessary for aneuploid
testing. The variation was therefore examined to see whether, if genetically closely related strains were crossed in standardised conditions, variation in the spontaneous frequency of aneuploidy would be sufficiently low to warrant the use of historical controls.

The distribution of the various types of aneuploid-containing asci was also examined to see if a standard spectrum of ascus types could be produced for control crosses, to allow comparison with the spectrum following aneuploid induction.

The possibility of extending the Sordaria aneuploid detection system to study aneuploidy in a second linkage group was brought about when mutant strains for the grey-6 locus on linkage group IV were received from Dr. H.L. K. Whitehouse. He reported that, in crosses of these mutants, asci with four black and four abortive spores were seen. It was decided to follow up this observation to see if these mutants could form the basis of an aneuploid detection system. This will subsequently be described as the grey-6 aneuploid detection system. The classification of ascus types, originally designed for the buff system, is also used for the grey-6 system.

The reasons for developing an aneuploid detection system using a second linkage group were as follows: A comparison could then be made between overall aneuploid frequencies for two linkage groups. More importantly, it could be determined whether the spectrum of meiotic
errors was similar or whether one chromosome was more susceptible to a particular meiotic error than the other. Once spontaneous aneuploidy had been studied for the second linkage group it would be possible to ask if both linkage groups responded in a similar way to inducing treatments. Auxotrophic markers on linkage group IV appeared suitable for the development of a random spore plating method to detect aneuploidy. It would be useful to be able to detect aneuploidy for the same linkage group by both ascus analysis and random spore plating.

1. The buff aneuploid detection system

   a) Classification of asci with aneuploid spores

As explained in the introduction black, or disomic, spores are found in various types of asci. From the spore sequence it is possible to infer the probable meiotic errors which gave rise to the asci. It is convenient to classify these asci into four specific types and a miscellaneous category. Throughout this thesis the following abbreviations will be used to describe spore phenotypes: +, wild type spores (black colour); -, spore colour mutant (buff or grey) and ab, abortive spores.

   Type 1 asci. These are asci with four black and four abortive spores and are the most frequently observed asci with aneuploid spores. The proposed origin of such asci is by non-disjunction or non-conjunction of homologous chromosomes at the first meiotic division, the black spores being disomic and the abortive spores presumably nullisomic.
These two possible meiotic errors are illustrated in fig. 3.1, where it can be seen that in both cases the expected resulting spore sequence is 4+:4ab, the black spores having two copies of linkage group II, the abortive spores none. As described in chapter 1 spores arising from non-conjunction should show no recombination, while spores arising through non-disjunction should show normal or even enhanced frequencies of recombination. Potentially this could be used to distinguish non-conjunction and non-disjunction and attempts to do so are discussed later in this chapter.

The effect of spindle overlap on spore sequence was discussed in chapter 1. An example of a type 1 ascus with a 2+:2ab:2+:2ab sequence is shown in figure 3.1. This probably arose through partial spindle overlap. Asci with this sequence could also arise through premature centromere division of both copies of linkage group II or second division non-disjunction at both poles, following crossing-over in the buff-centromere interval (see type 3 asci). As single events of this kind are detected infrequently, in comparison to first division non-disjunction or non-conjunction, it is extremely unlikely that double events make an important contribution to the frequency of type 1 asci. Asci with the spore sequence 2+:4ab:2+ and 2ab:4+:2ab have also been observed, although these are rare. One possible explanation for these is complete spindle overlap at the second meiotic
FIGURE 3.1 The origin and appearance of type 1 asci

Nonconjunction

or

Nondisjunction

s/o spindle overlap
division which has been shown to be rare (Berg, 1966). Complete spindle overlap would result in a 2:4:2 sequence for all centromere markers. Mating type can be used as a centromere marker for linkage group I. Two 2:4:2 type 1 asci were dissected and the black spores germinated and mating type tested. In both asci the sequence for mating type was 2:4:2. This was consistent with these asci having arisen through complete spindle overlap.

Type 2 asci: This class contains asci with two black, two abortive and four spore colour mutant spores in the sequence 2+:4- : 2ab or 2- :2+:2ab:2-. Examples of this ascus type are shown in figure 3.2. The simplest origin which can be ascribed to such asci is premature centromere division of one of the copies of linkage group II, at the first meiotic division. This is illustrated in figure 3.2. Partial spindle overlap has no effect on either of the spore sequences described above, because it switches the position of the central spore pairs. Following recombination in the buff-centromere interval fifty percent of premature centromere division events would not be detected. This will form only a small proportion of the total, as the centromere distance of the buff locus is 4 map units.

Type 3 asci: This class contains asci with two black, two abortive and four spore colour mutant spores in the sequence 2+:2ab:4-, 2ab:2+:4-, 2+:2-:2ab:2- and 2ab:2-:2+:2-. A simple explanation for asci with the first two spore sequences is non-disjunction at the second meiotic division
FIGURE 3.2 The origin and appearance of type 2 asci.
following crossing-over in the buff-centromere interval and this is illustrated in figure 3.3. This interval is approximately 4 map units, therefore only 8% of asci will contain recombinants, and only 8% of second division non-disjunction asci will be detected (however, see p.93 for a possible complicating factor). Spindle overlap can change the first two sequences in this class into the third and fourth sequences respectively. This is illustrated in figure 3.3. This results in the black and abortive spores being in the opposite halves of the ascus and these sequences could also arise by premature centromere division. Asci with any of these spore sequences are therefore ambiguous in origin. The 2+:2ab:4- and 2ab:2+:4- could arise through second division non-disjunction without spindle overlap or premature centromere division with spindle overlap while the opposite combination of error and spindle overlap could generate the 2+:2-:2ab:2- and 2ab:2-:2+:2- sequences.

Asci arising through premature centromere division would be expected to fall equally frequently into type 2 or type 3. The amount of second division non-disjunction should then be indicated by the difference between type 2 and type 3 asci. If the frequencies of type 2 and type 3 asci were equal this would indicate either no second division non-disjunction was occurring or none was being detected. This latter situation could arise in a cross where recombination was reduced, as detection of second division non-disjunction depends on crossing-over in the buff-centromere interval.
FIGURE 3.3 The origin and appearance of type 3 asci

Premature centromere division

or

Second division nondisjunction

s/o spindle overlap
Type 4 asci: These are asci which contain black spores but no abortive spores and include three different kinds of ascus, 2+:6-, 4+:4- and, more rarely 8+. There are two proposed origins for the 4+:4- asci. These are extra-replication of one copy of linkage group II or mitotic non-disjunction of linkage group II at the division preceding meiosis. Either of these events will result in there being three copies of linkage group II at the start of meiosis, that is, the ascus initial will be trisomic. It has to be proposed that the events leading to type 4 asci occur in the division preceding meiosis, and not earlier in the mitotic proliferation phase in perithecial development, as no clustering of type 4 asci has been observed. Lack of any clustering effect may be due to instability of disomic nuclei, as aneuploid nuclei are known to lose chromosomes readily to regain the haploid state (Pittenger, 1954).

When the ascus initial is trisomic a 4+:4- ascus is the result of two of the three possible chromosome combinations, when S6 and C70 go to the same pole (figure 3.4). If crossing-over occurs in the buff-centromere interval when S6 and C70 pair then in half the cases the resulting ascus will have 2+:6- spores, in which in addition to the two black disomic spores two of the buff spores will also be disomic. An 8+ ascus probably results from a double event, either extrareplication of both copies of linkage group II or mitotic non-disjunction of both copies, followed by appropriate segregation of the buff alleles. In this
FIGURE 3.4 The origin and appearance of type 4 ascospores

Extra replication or mitotic nondisjunction

Extra replication or mitotic nondisjunction + crossing-over
case the ascus initial will be tetrasomic.

**Miscellaneous.** Although types 1 - 4 form the majority of asci with aneuploid spores other types are seen and these are grouped under the heading miscellaneous. The most frequently observed of these others are asci with two black, two buff and four abortive spores. Explanations for such asci are more complex. One possibility is that they are type 1 asci in which there is maturation failure of one spore pair. Other possibilities are that the excess abortive spores could arise from simultaneous aneuploidy of another linkage group or from a defect other than nullisomy.

Occasionally other asci are found in which there are unequal numbers of black and abortive spores, but which could be simply explained on the hypothesis that one spore had failed to develop full pigmentation, for example an ascus with $3+:1-:4ab$ spores could be reclassified as type 1. Only a small number of asci were reclassified in this way. Other unusual asci which could not be explained by pigmentation failure generally had an excess of abortive spores, or large spores with fewer than eight in an ascus.

Occasionally black spores were detected, but the accompanying buff and/or abortive spores were not all clearly visible at x 400 magnification. In these cases the rosette was teased apart to obtain a clear view of the ascus. The success of this manipulation was very dependent on the state of the material. If it was very fragile and broke up easily, then dissection failed. Any ascus for which
spore content and sequence were not accurately determined was, therefore, unclassified.

**A gene dosage effect of the C70 allele**

It has recently been demonstrated that in homoallelic C70 x C70 crosses asci are found containing spores darker in colour than the normal C70 phenotype (Bond, unpublished). These spores are usually accompanied by abortive spores. By marking the chromosomes these dark spores were shown to be disomic and so presumably arose from a dosage effect in which two copies of the C70 allele, in the same nucleus, cause dark spores to be produced. The extent of the increase in pigmentation varies considerably, both between and within crosses, but occasionally is as dark as the colour produced by S6/C70 complementation. The possibility has to be considered that, in an S6 x C70 cross, an ascus which contains two C70 chromosomes will occasionally be scored as aneuploid. This will have only marginal effects on the proportions of type 1 and type 2 asci detected. However, more second division non-disjunction asci might be found than would have been predicted, as non-disjunction of C70 chromatids would sometimes give detectable black spores. In an attempt to determine how many type 3 asci were C70/C70 disomics, Bond (unpublished) carried out an ascus dissection experiment, to see what proportion of type 3 asci contained both an S6 and a C70 component. There have been a few cases where only a C70 component has been recovered but the numbers analysed are still too small to
give a definite proportion of C70/C70 disomics. This will probably vary between crosses because the maturity of the cross will be critical for detecting C70/C70 disomics. There will also be more type 4 asci detected than expected as two copies of C70 travelling to the same pole could produce a 4+:4- ascus. This gene dosage effect does not detract from the usefulness of the aneuploid detection system but makes the proportion of type 3 and 4 asci one would expect to detect less certain.

b) Spontaneous frequencies of aneuploidy

With one exception, which will be considered in Chapter 5, data from all the buff control crosses analysed in this work have been summarised in Table 3.1. This gives the total aneuploid frequencies for each cross and the numbers and frequencies of the different ascus types.

i) Total frequencies

The observed frequencies of aneuploid-containing asci ranged from $5.6 \times 10^{-4}$ to $12.4 \times 10^{-4}$. To test whether this set of data was homogeneous or heterogeneous $G_H$ tests were applied to the data. The outcome of the tests is summarised below. A line linking a group of numbers indicates that this group is homogeneous at the 0.05 probability level.

| Frequency $x 10^{-4}$ | 5.6 | 7.1 | 7.3 | 7.4 | 7.6 | 8.4 | 8.9 | 9.2 | 9.6 | 12.4 |

This shows that the data can be placed in two homogeneous groups, one including all frequencies but the highest, the other all frequencies but the lowest. The frequencies
TABLE 3.1 Number (and frequency x 10^{-4}) of asci containing black aneuploid spores in buff x buff crosses

<table>
<thead>
<tr>
<th>Ascus Type</th>
<th>Spore sequence</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4+4ab</td>
<td>31 (4.5)</td>
<td>28 (4.3)</td>
<td>23 (3.9)</td>
<td>27 (4.0)</td>
<td>30 (5.5)</td>
<td>33 (5.9)</td>
<td>30 (5.8)</td>
<td>29 (3.2)</td>
<td>28 (7.0)</td>
<td>31 (4.4)</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>2+4-12ab</td>
<td>2 (0.3)</td>
<td>3 (0.5)</td>
<td>5 (0.8)</td>
<td>4 (0.6)</td>
<td>1 (0.2)</td>
<td>2 (0.4)</td>
<td>1 (0.4)</td>
<td>4 (0.2)</td>
<td>0 (-)</td>
<td>3 (0.4)</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>2+-2ab</td>
<td>3 (0.5)</td>
<td>0 (0.2)</td>
<td>3 (0.5)</td>
<td>2 (0.3)</td>
<td>2 (0.4)</td>
<td>0 (-)</td>
<td>2 (0.4)</td>
<td>3 (0.3)</td>
<td>0 (-)</td>
<td>1 (0.1)</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>2+-2ab</td>
<td>3 (0.5)</td>
<td>6 (0.8)</td>
<td>3 (0.8)</td>
<td>1 (0.2)</td>
<td>3 (0.6)</td>
<td>4 (0.7)</td>
<td>5 (1.0)</td>
<td>2 (0.2)</td>
<td>5 (1.2)</td>
<td>1 (0.1)</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>4+4-</td>
<td>5 (0.7)</td>
<td>5 (0.8)</td>
<td>8 (1.3)</td>
<td>6 (0.9)</td>
<td>11 (2.0)</td>
<td>3 (0.5)</td>
<td>8 (1.5)</td>
<td>4 (0.5)</td>
<td>5 (1.2)</td>
<td>1 (0.1)</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>2+-2ab</td>
<td>1 (0.2)</td>
<td>5 (0.8)</td>
<td>3 (0.5)</td>
<td>2 (0.3)</td>
<td>0 (-)</td>
<td>2 (0.4)</td>
<td>0 (-)</td>
<td>1 (0.1)</td>
<td>0 (-)</td>
<td>0 (-)</td>
<td>14</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>**</td>
<td>1 (0.2)</td>
<td>0 (-)</td>
<td>2 (0.4)</td>
<td>4 (0.6)</td>
<td>0 (-)</td>
<td>5 (0.9)</td>
<td>1 (0.2)</td>
<td>3 (0.3)</td>
<td>7 (1.7)</td>
<td>3 (0.3)</td>
<td>28</td>
</tr>
<tr>
<td>Unclassified</td>
<td>***</td>
<td>4 (0.6)</td>
<td>3 (0.5)</td>
<td>1 (0.2)</td>
<td>4 (0.6)</td>
<td>3 (0.6)</td>
<td>1 (0.2)</td>
<td>3 (0.6)</td>
<td>4 (0.5)</td>
<td>5 (1.2)</td>
<td>10 (1.4)</td>
<td>38</td>
</tr>
<tr>
<td>Total aneuploids</td>
<td></td>
<td>50 (7.3)</td>
<td>50 (7.6)</td>
<td>50 (8.4)</td>
<td>50 (7.4)</td>
<td>50 (9.2)</td>
<td>50 (9.6)</td>
<td>50 (9.6)</td>
<td>50 (5.6)</td>
<td>50 (12.4)</td>
<td>50 (7.1)</td>
<td>500</td>
</tr>
<tr>
<td>Total asci screened</td>
<td></td>
<td>68450</td>
<td>65840</td>
<td>59650</td>
<td>67970</td>
<td>54210</td>
<td>55850</td>
<td>52190</td>
<td>89610</td>
<td>40280</td>
<td>70250</td>
<td>624300</td>
</tr>
</tbody>
</table>

* = black  
ab = abortive  
- = buff  
** Including asci with less than 8 spores and asci with an uneven number of black and abortive spores  
*** Asci for which the full sequences of spores was not determined, usually because the material was too fragile for further analysis. The majority of these had 2 black spores.
common to both groups could be considered as the normal range of control frequencies, showing some minor variation between crosses. The lowest and highest frequencies show that more extreme variation could be observed. The explanation for such variation is not clear. As will be seen in chapter 4 the cross with the high frequency of $12.4 \times 10^{-4}$, also showed very high frequencies of aneuploidy following chemical induction, consistent with this cross having a high base level of aneuploidy. The cross with the lowest frequency of aneuploidy, $5.6 \times 10^{-4}$, did not show lower levels of induction (see chapter 5).

The variation in spontaneous levels of aneuploidy is of interest when the factors controlling aneuploidy levels are considered. The fluctuations observed here must either be due to an uncontrolled environmental factor or to genetic differences in the isolates or both. As replicate crosses set up at the same time and subjected as far as possible to identical conditions showed no sign of variation, and as fresh isolates were used in all experiments, it is possible that the major deviations were due to genetic differences, despite the fact that isolates were always obtained from a stock cross.

Although there is variation between crosses it is still possible to use the system for aneuploid testing as this only depends on replicate crosses in an experiment having no major differences in frequency, which was previously found by Bond and McMillan (1979). The variation between crosses does prevent the use of historical controls which is
a disadvantage insofar as the system would be much more efficient if historical controls could be used. The analysis of control levels has produced a range of aneuploid frequencies, within which a control cross can be said to be behaving normally, that is around $7 \times 10^{-4}$ to $10 \times 10^{-4}$. Any control crosses which produce frequencies outside this range should be considered carefully, as there may be something unusual about the cross, although not necessarily so.

ii) **Analysis of the distribution of ascus types**

Any variation in the frequency of aneuploidy could be due to variation in the spectrum of asci with aneuploid spores or to an overall increase or decrease in frequency affecting all types of asci. Even within a group of crosses homogeneous for total aneuploidy, there could be variation in the spectra. In order to examine this histograms have been plotted showing the distribution of ascus types in crosses 1 - 10 (figure 3.5). The numbers shown in table 3.1 have been expressed as proportions of total asci containing aneuploid spores. This makes it easier to compare distributions without differences being masked by the variation in the overall frequency of aneuploidy.

One problem when comparing the distribution, or spectrum, of ascus types for any one experiment is, that the numbers of asci involved, except for type 1, are very small. This means that chance fluctuations in the spectra could occur due to sampling errors. As
FIGURE 3.5  Distribution of ascus types detected in experiments 1-10 (buff system)

M - miscellaneous  U - unclassified
comparisons are being carried out on proportions, if one ascus type increases, then others must form a decreased proportion of aneuploid-containing asci. The only class with sufficiently high numbers to justify statistical analysis was type 1. A $G_H$ test was therefore carried out on the type 1 data and this indicated that all the spectra were homogeneous with respect to type 1 asci ($G_H = 5.578$, $P > 0.25$).

Considering figure 3.5 it can be seen that type 3 asci usually formed a proportion greater than or equal to, the proportion of type 2 asci. This indicated that some second division non-disjunction was occurring. If only premature centromere division was contributing to type 3, no excess would be expected. Type 4 asci showed the most marked fluctuations, the proportion ranging from 0.22 to 0.02. Bond and McMillan (1979b) also noticed large fluctuations in type 4 asci, with proportions up to 0.61. The frequency of unclassified asci varied, and was a reflection of the quality of the material. As most of the unclassified asci contained two black spores this could explain some of the fluctuations in the proportions of type 2 and type 3, for example in experiment 10 eight of the ten unclassified asci had two black spores.

Experiments 8 and 9 had the most extreme values for total aneuploid frequency, but it is clear that this is not due to any major change in the spectra, but to a general effect on the level of aneuploidy.
The overall impression from the spectra is one of slight fluctuations, perhaps due to small numbers, but no major differences, with the possible exception of type 4. In order to obtain a standard spectrum for spontaneous aneuploidy, the aneuploid-containing asci were summed across experiments, to produce the spectrum illustrated in figure 3.6. This will be used for comparisons with treated crosses in later chapters.

iii) Detailed consideration of type 2 and type 3 asci

Although the numbers of type 2 and type 3 asci were small for any one experiment, summing ten experiments gives figures more reasonable for analysis. A comparison of type 2 and type 3 asci can be used to try to determine the contributions of premature centromere division at the first meiotic division and second division non-disjunction to aneuploidy in Sordaria. The simplest approach is to assume that premature centromere division gives rise equally often to type 2 and type 3 asci. Any excess of type 3 asci can then be attributed to second division non-disjunction. From the data illustrated in figure 3.6 the proportion of second division non-disjunction can be calculated as 0.1 - 0.05 which equals 0.05. The proportion of premature centromere division is then 0.1. From these figures it appeared that premature centromere division and second division errors contributed equally to type 3 asci.

Examination of the spore sequences of type 3 asci revealed some unexpected inconsistencies in the data. Type 3 asci can be subdivided into those with a 2:2:2:2 spore
FIGURE 3.6 Standard spectrum of spontaneous aneuploids for the buff detection system. n=500

Proportion of total aneuploid-containing ascites

Ascus type

miscellaneous - misc
unclassified - un
sequence and those with a 2:2:4 sequence (table 3.1). With no spindle overlap all 2:2:2:2 asci classed as type 3 would have arisen through premature centromere division and all 2:2:4 asci by second division errors. If we let the real proportion of premature centromere division asci in type 3 equal x, the real proportion of second division errors equal y and the proportion of asci with spindle overlap equal s then it follows that:

\[
\text{Observed 2:2:2:2} = (1 - s)x + sy \quad \text{Equation 3.1}
\]
\[
\text{Observed 2:2:4} = (1 - s)y + sx \quad \text{Equation 3.2}
\]

The proportion of asci with spindle overlap can be calculated from the numbers of 4+:4ab and 2+:2ab:2+:2ab asci (chapter 1). This is known to vary between crosses, but to be approximately 0.5. From the ten experiments reported here a value of 0.48 was calculated. Substituting a value of 0.5 for s into equations 3.1 and 3.2 leads to the expectation that the observed proportion of 2:2:2:2 asci would equal the observed proportion of 2:2:4 asci. The proportions found were 0.03 and 0.07 respectively (data in table 3.1), which could not be described as equal.

One explanation for the above results is that the proportion of spindle overlap asci is 0.5 but that for type 2 and 3 there is preferential discharge of spindle overlap asci. Preferential discharge has been considered at length by MacDonald and Bond (1974) and MacDonald (1976). They found some evidence that this occurred, although no conclusive proof was obtained. If it did occur and reduced the spindle overlap asci by a factor p then
equations 3.1 and 3.2 would become

Observed 2:2:2:2 \quad = (1 - s)x + s y

Observed 2:2:4 \quad = (1 - s)y + s x

This would explain unequal observed numbers of the subdivisions of type 3 asci, with s being approximately 0.5, provided \( x \neq y \). However, the numbers of type 2 asci suggested that \( x \) and \( y \) were approximately equal.

Another possible explanation is that, although \( s = 0.5 \) spindle overlap did not affect premature centromere division and second division non-disjunction asci equally. Assuming that premature centromere division asci have normal spindle overlap but that second division non-disjunction asci are less prone to spindle overlap by a factor, \( r \), then the equations become

Observed 2:2:2:2 \quad = (1 - s) + s r y

Observed 2:2:4 \quad = (1 - s r)y + s x

The observed frequencies are no longer expected to be equal even if \( s = 0.5 \) and/or \( x = y \).

As premature centromere division arises through lack of maintenance of sister chromatid pairing at the first meiotic division there is no reason to assume that asci in which this arose would have altered spindles at the second meiotic division. However, it is possible that, at least in some cases, second division errors may occur due to abnormal spindles at the second meiotic division, and these may preclude spindle overlap.
The discussion above shows that there are some inconsistencies in the data and so it is difficult to make any definitive statement about the proportions of second division non-disjunction asci. The best estimate that can be made is that based on the assumption that premature centromere division contributes equally to type 2 and type 3 asci. The estimated proportion of second division non-disjunction asci among detected aneuploid containing asci is then 0.05. The frequency of detected second division errors would depend on the overall aneuploid frequency in a cross.

The relative proportions of the occurrence of the various meiotic errors differ from the spectrum shown in figure 3.6, as equal percentages of all errors are not detected. Table 3.2 shows the percentage of each error which is detected and the relative proportions of the occurrence of each error. From this it appears that first division errors and second division errors occur in almost equal proportions, with much fewer pre-meiotic events. Premature centromere division makes only a small contribution to the proportion of first division errors.

The values in table 3.2 are based on a simplified calculation as the effect of C70/C70 gene dosage has not been considered.
TABLE 3.2

The proportions of various meiotic errors detected by ascus analysis using the buff system and the relative proportions with which such errors actually occur.

<table>
<thead>
<tr>
<th>Error</th>
<th>Undetected in</th>
<th>% detected</th>
<th>Observed Proportion</th>
<th>Real Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st division non-disjunction or non-conjunction</td>
<td>50% cases of crossing-over in buff-centromere interval</td>
<td>96%</td>
<td>0.58</td>
<td>0.39</td>
</tr>
<tr>
<td>Premature centromere division</td>
<td>do.</td>
<td>96%</td>
<td>0.10</td>
<td>0.07</td>
</tr>
<tr>
<td>2nd division errors</td>
<td>all cases when no crossing-over in buff centromere interval</td>
<td>8%</td>
<td>0.05</td>
<td>0.42</td>
</tr>
<tr>
<td>extrareplication or mitotic non-disjunction</td>
<td>1/3 cases of no crossing-over and 1/6 cases of crossing-over in buff centromere interval</td>
<td>68.3%</td>
<td>0.14</td>
<td>0.13</td>
</tr>
</tbody>
</table>
c) **Summary of spontaneous aneuploidy observed using the buff aneuploid detection system**

This analysis has shown that

i) A variety of meiotic errors generate aneuploidy.

ii) There is some heterogeneity in the frequency of aneuploidy, which is not great, in that 8 out of 10 crosses form a homogeneous group, but it is probably sufficient to prevent the use of historical controls. The difference in the extreme values (approximately x 2) is minor in comparison to control variation found by Griffiths (1979) in Neurospora, who reported thirty-fold differences in controls.

iii) By summing the data on types of aneuploidy in all control crosses, a general picture emerged, where the observed frequency of errors of non-conjunction and non-disjunction at the first meiotic division comprise the greatest proportion (0.68) of aneuploid generating events.

iv) The proportions of detected aneuploid-containing ascis due to premature centromere division at the first meiotic division and to second division non-disjunction were calculated as 0.10 and 0.05 respectively. It appeared that spindle overlap may be reduced in ascis in which second division non-disjunction had occurred.

v) From calculations of the relative proportions with which aneuploid events actually occur first and second division errors appear to arise with almost equal frequencies.
2. Development of the grey aneuploid detection system

a) Preliminary analysis

Five mutants at the grey-6 locus were available: YS121, RW25, C31, YS18 and B9. All five produce a grey spore colour, the first three being indistinguishable from each other. YS18 has a darker grey phenotype and B9 produces paler grey spores than the other three. In order to ascertain which cross gave the best complementation, that is the most easily detectable disomic spores, crosses shown in table 3.3 were analysed. This was obviously a subjective analysis of the 'blackness' of spores, and table 3.3 gives an indication of the extent of complementation observed.

YS18 and B9 were immediately ruled out for use in aneuploid screening. Complementation involving B9 was too weak to allow easy detection of aneuploidy, given the method used. The dark grey colour of YS18 spores meant that the contrast between these and the black aneuploid spores was not sufficient to allow the black spores to stand out clearly. For either YS18 or B9 crosses the aneuploid frequencies could not be stated with confidence. The remaining three interallelic crosses RW25 x YS121, RW25 x C31 and YS121 x C31 all gave easily detectable, darker aneuploid spores but in no case did the complementation restore the wild type colour as fully as in the buff system. Despite this it was possible to score aneuploid-containing asci reliably. Although complementation between mutants
TABLE 3.3
Cresses of grey-6 mutants and ease of detection of aneuploid spores

<table>
<thead>
<tr>
<th></th>
<th>YS121</th>
<th>RW25</th>
<th>C31</th>
<th>YS18</th>
<th>B9</th>
</tr>
</thead>
<tbody>
<tr>
<td>YS121</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>RW25</td>
<td>+</td>
<td>+++</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>C31</td>
<td>-</td>
<td></td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

+ → +++ increasing pigmentation
n.s. not scoreable
- no darker spores observed
was not always sufficient to allow scoring for aneuploidy, it did appear to occur in all the interallelic crosses shown in table 3.3. It is unusual to find that all alleles at a locus complement. In addition, disomic spores in homoallelic crosses of RW25 x RW25 and YS121 x YS121 were also dark, this reflecting a dosage effect similar to that found in C70 x C70 crosses. These dosage effects for RW25 and YS121 were unlikely to have a large effect on the proportion of aneuploid-containing asci detected as the degree of colour in the spores generally appeared to be less than that in interallelic crosses.

From preliminary crosses it was determined that all ascus types described for the buff system were observed in grey-6 crosses. In order to demonstrate that the dark spores in ascus types 1, 2 and 3 were aneuploid, a sample of such asci was dissected and the spores crossed to wild type. Recombinant spores would produce asci with eight black spores in such crosses, while aneuploid spores would give heterokaryotic cultures and asci would segregate for grey spore colour. Asci were dissected from each of the three crosses and in total at least one of each ascus type 1, 2 and 3 was analysed. All black spores were found to be aneuploid.

The major difference between the buff and grey-6 systems was that in each of the grey-6 crosses a high frequency of asci with two black and six grey spores was observed. These were therefore considered in more detail.

b) Analysis of 2+:6- asci

Table 3.4 shows the data obtained for the frequency of 2+:6- asci in grey-6 x grey-6 crosses. The high frequencies
TABLE 3.4

Number (and frequency %) of asci with 2 black: 6 grey and 4 black: 4 grey spores in \textit{grey-6} x \textit{grey-6} crosses

<table>
<thead>
<tr>
<th>Alleles Crossed</th>
<th>Asci Screened</th>
<th>2 black: 6 grey</th>
<th>4 black: 4 grey</th>
</tr>
</thead>
<tbody>
<tr>
<td>RW25 x C31</td>
<td>69640</td>
<td>1207 (1.7)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>63790</td>
<td>1181 (1.8)</td>
<td>6++</td>
</tr>
<tr>
<td></td>
<td>9960</td>
<td>211 (2.1)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>9770</td>
<td>239 (2.4)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>9460</td>
<td>102 (1.1)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>7370</td>
<td>112 (1.5)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8630</td>
<td>103 (1.2)</td>
<td>2+</td>
</tr>
<tr>
<td></td>
<td>6110</td>
<td>120 (1.9)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>19040</td>
<td>137 (0.7)</td>
<td>3</td>
</tr>
<tr>
<td>C31 x YS121</td>
<td>35020</td>
<td>419 (1.2)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>6980</td>
<td>59 (0.8)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>32500</td>
<td>258 (0.8)</td>
<td>5</td>
</tr>
<tr>
<td>RW25 x YS121</td>
<td>135790</td>
<td>79 (0.06)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>8390</td>
<td>8 (0.10)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>6360</td>
<td>5 (0.08)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9800</td>
<td>5 (0.05)</td>
<td>4+</td>
</tr>
</tbody>
</table>

+ One ascus with large black spores
++ Two asci with large black spores
with which these asci were found, compared to other asci with black spores, and the frequency varying depending on which alleles were crossed, suggested that these asci were likely to be due, not to aneuploidy, but to recombination. This is supported by the observation that 4+:4- asci occurred at a much lower frequency than 2+:6- asci, as would be expected if their origin was from recombination events. The opposite relationship would hold if the asci had aneuploid origins.

More direct evidence comes from tests on the black spores obtained on dissection of 2+:6- asci. Black spores from twelve such asci found in an RW25 met- x C31 cross were tested and shown to be recombinant and not aneuploid. These dissections could also be used to order the grey-6 alleles because the flanking met marker was present. The black spores would be met- if the order were met C31 RW25 and met+ if the order were met RW25 C31. Ten out of twelve were met- suggesting the order met C31 RW25. The remaining two were met+ and are most likely to have arisen by gene conversion of C31 since double crossovers in the short intervals involved are very unlikely.

With such high frequencies of crossing-over between the different spore colour mutants it may be that the grey-6 locus is not simply one gene, but two closely linked genes with C31 located in one and RW25 in the other. It is clear from table 3.4 that, despite variation in frequencies, each type of cross has its own band of
frequencies of 2+:6- asci. These can be used to produce an order for the three mutants, assuming the lower the frequency, the closer the mutants. The much lower frequencies in RW25 x YS121 crosses suggests that these two mutants could be alleles and that the 2+:6- asci in RW25 x YS121 crosses are gene conversion octads. As the crosses had no flanking markers this hypothesis was not tested. The possibility of C31 being a mutation of a different gene than RW25 and YS121 would mean that the observation of complementation between all the grey-6 mutants would not be so unusual, as they would not then all be alleles.

The high frequency of 2+:6- asci caused a problem of classification of the 4+:4- asci, which would otherwise have been attributed to type 4 origins, that is extra-replication or mitotic non-disjunction. Such asci often have large spores, although this is not always the case. The 4+:4- asci observed in grey x grey crosses could have arisen through a double recombination or gene conversion event. When analysed two 4+:4- asci with large spores turned out to be aneuploid, and a single 4+:4- ascus with small spores was not. However spore size could not be used as a definite criterion of whether or not a 4+:4- ascus had aneuploid origins, as it was known from the buff system that aneuploid black spores in 4+:4- asci are not necessarily enlarged.

The high frequency of 2+:6- asci was potentially very useful as these could then be used to monitor the
effects of inducing agents on recombination frequency or chromosome pairing.

c) **Spontaneous frequencies of aneuploidy**

Fresh isolates of RW25, YS121 and C31 were obtained from stock crosses and the three mutants were intercrossed. Reciprocal crosses were carried out to determine which isolates were the best females. All YS121 isolates were found to be female sterile, while C31 and RW25 were equally good female parents.

(i) **Total frequencies**

The fact that heterogeneity was found using genetically closely related isolates of buff spore colour mutants suggested that heterogeneity could also be expected in crosses of the different *grey-6* alleles. Table 3.5 shows the data obtained for ascus types 1, 2 and 3. For the reasons given above type 4 asci were not scored. The frequency of aneuploidy in RW25 x YS121 crosses was very low and there was insufficient material to count to 50 aneuploid-containing asci. The data in Table 3.5 for RW25 x YS121 is the sum of two crosses, one of which yielded only eight type 1 asci.

It is clear that there is heterogeneity among the frequencies of aneuploidy, the range being $2.1 \times 10^{-4}$ to $14.3 \times 10^{-4}$. This is a wider range than that observed among buff crosses, although it is still considerably narrower than that observed by Griffiths (1979). The RW25 x YS121 crosses have a very low frequency compared to the others. The data is not sufficient to determine
<table>
<thead>
<tr>
<th>Ascus Type</th>
<th>Spore sequence within ascus</th>
<th>RW25 x C31 1</th>
<th>RW25 x C31 2</th>
<th>C31 x YS1 2F 3</th>
<th>RW25 x YS121 4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(4^+; 4^{ab} ) (2^+; 2^{ab} ) (2^+; 2^{ab} ) (2^+; 2^{ab} )</td>
<td>31 (4.5)</td>
<td>36 (5.6)</td>
<td>33 (9.4)</td>
<td>18 (1.3)</td>
<td>118</td>
</tr>
<tr>
<td>2</td>
<td>(2^+; 2^+; 2^+; 2^+ ) (2^+; 2^+; 2^+; 2^+ ) (2^+; 2^+; 2^+; 2^+ ) (2^+; 2^+; 2^+; 2^+ )</td>
<td>5 (0.7)</td>
<td>9 (1.4)</td>
<td>4 (1.1)</td>
<td>1 (0.07)</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>(2^+; 2^+; 2^+; 2^+ ) (2^+; 2^+; 2^+; 2^+ ) (2^+; 2^+; 2^+; 2^+ ) (2^+; 2^+; 2^+; 2^+ )</td>
<td>2 (0.3)</td>
<td>3 (0.5)</td>
<td>5 (1.4)</td>
<td>2 (0.15)</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>(2^+; 2^+; 2^+; 2^+ ) (2^+; 2^+; 2^+; 2^+ ) (2^+; 2^+; 2^+; 2^+ ) (2^+; 2^+; 2^+; 2^+ )</td>
<td>See Table 3.4</td>
<td>See Table 3.4</td>
<td>See Table 3.4</td>
<td>See Table 3.4</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>*</td>
<td>3 (0.4)</td>
<td>0 (-)</td>
<td>3 (0.9)</td>
<td>5 (0.4)</td>
<td>11</td>
</tr>
<tr>
<td>Unclassified</td>
<td>**</td>
<td>0 (-)</td>
<td>0 (-)</td>
<td>0 (-)</td>
<td>0 (-)</td>
<td>0</td>
</tr>
<tr>
<td>Total Aneuploids</td>
<td></td>
<td>50 (7.2)</td>
<td>50 (7.8)</td>
<td>50(14.3)</td>
<td>26 (1.9)</td>
<td>176</td>
</tr>
<tr>
<td>Total Asci Screened</td>
<td></td>
<td>69640</td>
<td>63790</td>
<td>35020</td>
<td>135790</td>
<td>304240</td>
</tr>
</tbody>
</table>
whether the range of frequencies observed here is due to variation similar to that observed in *buff x buff* crosses, or whether the different *grey-6* mutants arose in isolates in which the fourth chromosomes had characteristic non-disjunction frequencies.

The low frequency of aneuploidy in RW25 x YS121 crosses suggests that these crosses, or alternatively crosses involving C31, may be atypical for linkage group IV. One possible explanation is that the chromosome carrying C31 has a small structural rearrangement, so that pairing with a non-homologous copy of linkage group IV leads to higher frequencies of non-disjunction. More extreme examples of chromosome rearrangements affecting aneuploidy are known for linkage group II (Bond, unpublished and Chapter 6). The idea that the C31 carrying chromosome is associated with increased aneuploidy is a testable hypothesis. In a cross of C31 x wild type there will be some 2+:6- gene conversion asci. Two of the grey spores will now have the C31 mutant in a different chromosome background. If crossed to RW25 and YS121, these grey spores should now give reduced levels of aneuploidy, if the hypothesis was correct.

There was no reason to predict that spontaneous aneuploidy frequencies would be similar or different for linkage group II or IV. The observation is that, at least for RW25 x C31 crosses, they are very similar.
(ii) The distribution of ascus types

If the variation in aneuploid frequency observed in crosses of the different alleles was due to other genetic differences in the crosses, then this could be brought about by effects on one particular ascus type. The aneuploid spectra for the various crosses are illustrated in figure 3.7a-d. It should be borne in mind that some of the numbers on which these are based are very small, especially for RW25 x YS121. Type 4 are absent from the spectra because of the complication arising from the high frequency of 2+:6- ascis. Apart from this the spectra are very similar to those obtained from buff x buff data. The major class is type 1 asci and type 3 are generally found in excess of type 2 asci. The RW25 x YS121 crosses had a very high proportion of miscellaneous asci, which were all of the type 2+:2-:4a/b. These formed a very high proportion for this normally rare ascus type, even when the small numbers involved were considered. One possibility is that such asci were really type 1, with complementation failing in one spore pair. There is no obvious reason why, if the explanation is a failure of pigmentation, the failure should affect spore pairs, rather than individual spores. If this were the case a high frequency of miscellaneous types with an odd number of black spores would be expected, and this was not found. The other explanations proposed earlier to explain this ascus class invoke a double non-disjunction event or non-disjunction plus unrelated spore abortion. These seem unlikely in a cross with a very low frequency
FIGURE 3.7 Distribution of ascus types detected in grey-6 crosses

a) C31×YS121 \( (n=50) \)

b) RW 25×YS121 \( (n=26) \)

c) RW 25×C31 \( (n=50) \)

d) RW 25×C31 \( (n=50) \)

e) RW 25×C31 \( (n=100) \)

- M miscellaneous
- U unclassified

Standard spectrum of spontaneous aneuploids for the grey-6 detection system
of aneuploidy and no suggestion of an unusually high frequency of spore abortion. It is therefore not clear how such asci arose. The numbers of each ascus type observed, other than of type 1, in any individual heteroallelic cross, were too small to justify further analysis.

In order to compare the occurrence of the different meiotic errors for the two linkage groups, the linkage group IV data were summed (figure 3.7e). Again type 3 asci were in excess of type 2, indicating some second division non-disjunction was occurring. There is no strong evidence from the grey data of an inequality in the numbers of 2:2:2:2 and 2:2:4 asci within type 3.

As for linkage group II, the proportions of asci due to premature centromere division and second division errors could be calculated and these figures used to determine the relative proportions with which the various meiotic errors occurred. The correction factors are slightly different from those used for linkage group II because the grey-centromere distance is 5 map units. The results are shown in table 3.6. For linkage group IV there appears to be a smaller proportion of second division errors than for linkage group II, with an increased proportion of premature centromere division at the first meiotic division.

These conclusions can, however, only be tentative, due to the small numbers involved. More data are necessary to confirm that there is a difference between linkage group II and IV in the proportions with which the various meiotic errors occur.
<table>
<thead>
<tr>
<th>Error</th>
<th>% detected</th>
<th>Observed Proportion</th>
<th>Real proportion occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st division non-disjunction or non-conjunction</td>
<td>95%</td>
<td>0.72</td>
<td>0.49</td>
</tr>
<tr>
<td>Premature centromere division</td>
<td>95%</td>
<td>0.23</td>
<td>0.16</td>
</tr>
<tr>
<td>2nd division errors</td>
<td>10%</td>
<td>0.05</td>
<td>0.35</td>
</tr>
</tbody>
</table>

The proportions of the various meiotic errors detected by ascus analysis using the grey-6 system and the relative frequencies of their occurrence.
(iii) Selecting a grey-6 x grey-6 cross for further experiments.

It was decided to use RW25 x YS121 for further experiments with the grey-system, because the low frequency of 2+;6- ascii made the crosses simpler to score. The disadvantage of 2+;6- ascii occurring at a high frequency was that scoring time was increased, because of having to ensure that the ascus type was diagnosed correctly by transferring to a higher magnification, and if necessary, by teasing apart the rosette containing the ascus. However, following reisolation of the RW25 and YS121 strains, crosses were completely unscoreable, the extra pigmentation in aneuploid spores being markedly reduced. Previously some minor differences in the degree of pigmentation had been noted between crosses, but had not affected scoring, and had been attributed to differences in maturity of crosses.

Now, before proceeding further, it was decided to carry out an analysis of crosses of fresh isolates of all three mutants, with and without auxotrophic markers, and harvested at various stages after crossing. All crosses were chemically treated with p-fluorophenylalanine (see Chapter 4 for a full analysis of this treatment, which induced aneuploidy and therefore allowed more rapid scoring) and approximately thirty rosettes per cross were examined. Crosses were harvested on days 10, 11 and 12 and an improvement in dark spore colour was found with
increasing maturity of crosses of all mutants. Although later harvests were more easily scored the differences were not sufficient to explain the impossibility of scoring the RW25 x YS121 cross mentioned above.

Comparing crosses not marked with auxotrophic mutants, all C31 x RW25 crosses were similarly easy to score. C31 x YS121 crosses were scoreable, with an occasional doubtful ascus, especially at earlier harvest times. Different isolates of RW25 x YS121 gave different results. Sometimes crosses were possible to score at all times of harvesting, others were very difficult or impossible. Of the crosses marked with auxotrophic mutants, only RW25 x C31 were successful. No differences in spore colour were observed, compared to unmarked crosses. All YS121 isolates showed poor fertility.

Despite the high frequency of 2+:6- asci in C31 x RW25 crosses it was decided to use these mutants in further studies with the grey-6 system, as the advantages of ease of detection of dark spores, and good fertility, outweighed the disadvantages of 2+:6- asci.

**d) Summary of the studies on the grey aneuploid detection system.**

The complementing mutants RW25 and C31 at the grey-6 locus on linkage group IV do provide a suitable aneuploid detection system. The overall frequency of aneuploidy and the spectrum of aneuploid types observed were very similar to those in buff x buff crosses. The grey-6 system has no advantages over the buff system in aneuploid detection, but potentially it is a useful addition to it.
The fact that the grey-6 alleles recombine with an appreciable frequency to produce 2+6- ascis means that grey crosses can be used to test whether an inducing agent increases aneuploidy through affecting chromosome pairing and/or recombination.

The grey-6 locus showed several interesting features. The apparent complementation of all five alleles at one locus was unusual, as was the high frequency of recombination between alleles. It could be that the grey-6 locus is in fact two closely linked gene loci, mutations at either giving grey spore colour. If true then from the data here it would be expected that RW25 and YS121 were mutations at one locus and C31 at the other.

3. Comparison of the relative proportions of meiotic errors occurring in Neurospora and Sordaria.

The relative proportions of the various meiotic errors occurring in Neurospora can be obtained from the data of Threlkeld and Stoltz (1970), who studied aneuploidy involving linkage group VI. Their system allowed detection of 98% first division errors but only 4% second division errors. Asci with black but no abortive spores were not recorded, therefore the frequency of extra-replication or mitotic non-disjunction is not known. The overall frequency of aneuploidy detected was approximately $3.3 \times 10^{-3}$, higher than any values obtained for spontaneous aneuploidy in Sordaria. They detected 76 asci with aneuploid spores of which 28 were attributed to first division non-disjunction or non-conjunction, 45 to
premature centromere division, and 3 to second division errors. Allowing for the differences in rates of detection the relative proportions of the occurrence of these errors were 0.19 first division non-disjunction or non-conjunction, 0.31 premature centromere division and 0.50 second division errors. The Sordaria and Neurospora data are very different, both in the degree of excess of second division errors, and also in the proportions of premature centromere division asci. While some of the differences in second division errors may be attributed to the small numbers involved, the same cannot be said for premature centromere division. Neurospora linkage group VI seems much more prone to this error than either linkage group studied in Sordaria.

4. Investigating non-conjunction and non-disjunction at the first meiotic division

Type 1 ascis can arise through either non-disjunction or non-conjunction of homologous chromosomes at the first meiotic division. These two alternatives can be distinguished by the recombination frequency in aneuploid spores, no recombination being possible if non-conjunction occurs, and non-disjunction either not affecting or enhancing recombination frequency. A sensitive test for distinguishing non-disjunction and non-conjunction would ideally have a chromosome, well marked along its entire length. This would permit the unambiguous detection of the origin of any $4+4ab$ ascus. Such a test is not possible in
Sordaria, due to lack of markers on linkage group II or linkage group IV. In the absence of markers along the entire chromosome, a loosely linked marker would permit the determination of the proportion of non-recombinant meioses although multiply recombinant meioses could not be detected.

Most markers known in Sordaria brevicollis are centromere linked and so unsuitable. If a mutant is found which is not centromere linked, then it is very difficult to discover on which chromosome it is located. Two unmapped markers were available in Sordaria, lys and met-6 which could not be assigned to any linkage group by conventional tests. One method, used in yeast to determine chromosome linkage of various markers is aneuploid mapping (Parry and Cox, 1970; Mortimer and Hawthorne, 1973 and Culbertson and Henry, 1973).

Using this method linkage is inferred when the unmapped marker shows the same aberrant segregations in crosses as markers known to be disomic in the parent strain. It was therefore decided to attempt aneuploid mapping in Sordaria for the lys and met-6 loci. Figure 3.8 illustrates the various possibilities for segregation of the markers in 4+:4ab asci. The buff system is used as an example but the same arguments apply for the grey-6 system. It is clear that, assuming recessivity of lys and met-6, asci in which the four black spores are auxotrophic prove no linkage and asci with four prototrophic spores, which yield requirers, on crossing to wild type, indicate
FIGURE 3.8(a) Aneuploid mapping using the buff system — Lys unlinked to buff

(i) no cross-overs between Lys and the centromere

resulting ascus

If the *Lys* and the *lys* chromosomes segregated in the opposite orientation—result *4 lys*⁺ black *4 lys*⁻ abortive

(ii) single cross-over between Lys and the centromere
FIGURE 3.8(b) Aneuploid mapping using the buff system — lys linked to buff

(i) no crossovers between buff and lys

\[ \text{S6}\quad\text{C70 lys} \]
\[ \text{1st div. non-disjunction} \]

\[ \text{resulting ascus} \]
\[ 4\ \text{lys}^+\ \text{black} \]
\[ 4\ \text{abortive} \]

(ii) single cross-over between buff and lys

\[ \text{S6}\quad\text{C70 lys} \]
\[ \text{1st div. non-disjunction} \]

\[ \text{2 lys}^+\ \text{black} \]
\[ 2\ \text{lys}^-\ \text{black} \]
\[ 4\ \text{abortive} \]

\[ \text{OR} \]
\[ 4\ \text{lys}^+\ \text{black} \]
\[ 4\ \text{abortive} \]
linkage. The argument for linkage assumes that the black spores are not simultaneously disomic for another linkage group. The fact that the proportions of non-disjunction and non-conjunction asci are not known does not affect the establishment of linkage.

Double mutants were constructed of each auxotroph with C70 and C31 and crossed to S6 and RW25 respectively. Dissection of 4+:4ab asci from all four crosses showed that neither lys or met-6 were linked to linkage group II or linkage group IV. In both crosses involving lys, asci with four black lys⁻ spores were found. In the crosses involving met-6, asci with four black, prototrophic spores did not yield auxotrophs in crosses to wild type, in analysis of fifty spores picked from five different rosettes.

As no linkage was inferred from aneuploid mapping, the problem of whether or not black spores were simultaneously disomic for other chromosomes did not arise.

Investigations into the proportion of 4+:4ab asci, arising due to non-disjunction, could not be carried out, as no suitable markers were available. Filtration enrichment and resistance mutant hunts did not yield any other suitable markers.

5. Summary

Two aneuploid detection systems are available in Sordaria brevicollis, one based on linkage group II spore colour mutants, the other on linkage group IV spore colour mutants. Both systems allow detection and
identification of various meiotic errors, and a characteristic spectrum of ascus types is produced. Although there is heterogeneity in the overall frequency of aneuploidy for each linkage group, the spectra show no major differences.

Various analyses of the linkage group II and IV data have been presented, to determine the relative proportions of the different meiotic errors and to try to resolve the ambiguities among type 3 asci. The analyses suggest that for linkage group II first and second division errors arise with almost equal frequencies, but that for linkage group IV second division errors appeared to form a smaller proportion of total errors, with a corresponding increase in premature centromere division at the first meiotic division. The analyses could be considered premature as often only small numbers of each ascus class, other than type 1, were available. However, they have been shown to illustrate the potential of the systems. They demonstrate the kinds of information that could be obtained, which would allow more definite conclusions, given more data.

The high frequency of recombination between the grey-6 spore colour mutants means that the grey-6 system can be used to monitor the effects of inducing agents, on chromosome pairing and recombination. There were some indications that the grey-6 locus may not be a simple gene but that two functional genes may be present, mapping very close together, although the evidence is slim.
Aneuploid mapping of unmapped auxotrophs did not show linkage to either linkage group II or IV and so analysis of the contribution of non-conjunction to type 1 asci could not be carried out.
CHAPTER 4: ANEUPLOID INDUCTION BY PARAFLUOROPHENYLALANINE

1. Introduction

The amino acid analogue parafluorophenylalanine (pFPA) has been reported to induce chromosome loss in Aspergillus niger (Lhoas, 1961) and Aspergillus nidulans (Morpurgo, 1961) and has been shown to induce aneuploidy in Neurospora crassa (Griffiths and Delange, 1977). Treatment with pFPA was used as an initial test of the Sordaria buff aneuploid detection system (Bond and McMillan, 1979a,b) and this work demonstrated that the system was sensitive to pFPA. The results suggested that there may have been a disproportionate increase in asci with two black and two abortive spores, compared to type 1 asci. However, in the analysis of the effect of pFPA, type 2 and type 3 asci were not distinguished. Further work was therefore necessary, to determine if this disproportionate increase in asci with two black and two abortive spores was real, and, if so, was the effect on premature centromere division or second division non-disjunction or both? If the main effect was on premature centromere division, then type 2 asci would be disproportionately increased, the effect on type 3 being diluted by the presence of second division non-disjunction asci in this class. However, if the second meiotic division was more sensitive to pFPA induction, then there would be a disproportionate increase in type 3 asci. This would also occur if pFPA increased recombination, as this would cause detection of a greater proportion of second division errors.

As two aneuploid detection systems are available in Sordaria, with characterised spectra for the distribution
of spontaneous aneuploids, it was possible to ask if the
effect of pFPA was linkage group specific, by examining
dose response curves and the spectra of ascus types, of
both the buff and grey-6 systems. Crosses were set up
for each system and treated with pFPA at various concent-
rations. Information from a series of crosses carried
out at higher doses was pooled, to give more data to analyse
the effects of pFPA on spectra.

Treatment of crosses

Treatment was as described by Bond and McMillan (1979a).
2.5 ml pFPA solution was pipetted over each plate, immediately
after fertilization of the crosses. Control plates were
treated with 2.5 ml distilled water. Plates were incubated
upright, overnight, and then any excess liquid was poured
off and the plates were incubated in the inverted position
until mature. The only variation in this procedure was
when applying pFPA at various times before fertilization.
pFPA was obtained from Sigma, product no. F5251.

2. Results

At the outset there were three main questions to be
asked of treatment with pFPA, along with a comparison of
the response of the buff and grey-6 systems in each case.
These questions were:

a) Does the treatment affect the overall frequency of
aneuploidy?

b) Is there a dose related effect?

c) Is the spectrum of aneuploid types altered? If so,
which class shows the disproportionate effect?
a) **Does the treatment affect the overall frequency of aneuploidy?**

Table 4.1 shows the results obtained in all pFPA treated crosses throughout the project, using concentrations of 10 mg/l and 20 mg/l, treating both buff and grey-6 crosses. In all cases the frequencies were based on counting fifty asci with aneuploid spores. Assuming that the frequency of aneuploidy in controls would be about $8 \times 10^{-4}$, then counting to fifty would be sufficiently sensitive to pick up an increase to a value half as much again. That is an increase to $12 \times 10^{-4}$ would give a 't' value significant at the 0.05 probability level. As tests for induction were always repeated the sensitivity of the system would actually be greater than this.

It is clear that pFPA caused an increased frequency of aneuploidy, although the level of increase was variable. To test the statistical significance of the effect of pFPA, a 't' test for paired differences was used, so that each induction was compared to its control. In all cases the increases were significantly different from zero, with the probability levels being less than 0.01 for buff crosses at both 10 and 20 mg/l and less than 0.05 for grey-6 crosses at 20 mg/l. This confirms the observation by Bond and McMillan (1979a,b) that pFPA induced aneuploidy in the buff system. Further to this it shows that induction by pFPA is not linkage group specific, as induction was observed using the grey-6 system.

Often counts for pFPA treated crosses were taken from
<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Controls</th>
<th>Treatments</th>
<th>'t' for paired differences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freq. x10^-4</td>
<td>Total Asci</td>
<td>Freq. x10^-4</td>
</tr>
<tr>
<td>Buff</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.3</td>
<td>68450</td>
<td>21.5</td>
</tr>
<tr>
<td></td>
<td>7.6</td>
<td>65840</td>
<td>22.9</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
<td>59650</td>
<td>26.9</td>
</tr>
<tr>
<td>4</td>
<td>7.4</td>
<td>67970</td>
<td>34.1</td>
</tr>
<tr>
<td>8</td>
<td>5.6</td>
<td>89610</td>
<td>19.4</td>
</tr>
<tr>
<td>Buff</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8.4</td>
<td>59650</td>
<td>54.2</td>
</tr>
<tr>
<td>5</td>
<td>9.2</td>
<td>54210</td>
<td>55.7</td>
</tr>
<tr>
<td>9</td>
<td>12.4</td>
<td>40280</td>
<td>91.2*</td>
</tr>
<tr>
<td>10</td>
<td>7.1</td>
<td>70250</td>
<td>85.5</td>
</tr>
<tr>
<td>a</td>
<td>7.2</td>
<td>69640</td>
<td>36.9</td>
</tr>
<tr>
<td>b</td>
<td>7.2</td>
<td>69640</td>
<td>59.4</td>
</tr>
<tr>
<td>R</td>
<td>7.8</td>
<td>63790</td>
<td>73.7</td>
</tr>
</tbody>
</table>

* 100 aneuploid-containing asci counted.
a single replicate, but if more than one was used, no difference was noted between replicates (see also Bond and McMillan, 1979a). However in the course of scoring the replicates of RW25 x C31 cross 2 it became obvious that the levels of induction on each plate were different. Each replicate was therefore counted until fifty aneuploid-containing asci had been observed, and the results are recorded as cross 2a and 2b in table 4.1. It is clear that the overall levels of induction are different in each case. One possible reason for such variability is that, as treatment is carried out immediately post-fertilization, the crosses are still damp, and if some water inadvertently remained, this could dilute the pFPA solution. While care was taken to prevent this occurrence, this may be a case where a plate with excess water remaining, went undetected.

b) Is there a dose related effect?

Table 4.2 shows the results obtained following pFPA treatment of both buff and grey-6 crosses, with a range of concentrations from 1-20 mg/l, and the results are presented graphically in Figure 4.1. For grey-6 crosses treated with 1 mg/l pFPA only twenty-five aneuploid-containing asci were recorded, due to poor material. To compare control and treated values when only one result was available, a simple t-test, based on a Poisson distribution was used. For both linkage groups the increases in aneuploid frequencies following treatment with 5 mg/l pFPA and above, were statistically significant (P < 0.01).
TABLE 4.2. Dose response of aneuploid induction by p-fluorophenylalanine.

<table>
<thead>
<tr>
<th>pFPA conc. mg/l</th>
<th>Buff crosses</th>
<th>Grey-6 crosses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freq. x10^-4</td>
<td>Total Asci</td>
</tr>
<tr>
<td>0</td>
<td>8.4</td>
<td>59650</td>
</tr>
<tr>
<td>1.0</td>
<td>10.8</td>
<td>46190</td>
</tr>
<tr>
<td>5.0</td>
<td>17.2</td>
<td>29000</td>
</tr>
<tr>
<td>10.0</td>
<td>26.9</td>
<td>18590</td>
</tr>
<tr>
<td>15.0</td>
<td>23.7</td>
<td>21090</td>
</tr>
<tr>
<td>20.0</td>
<td>54.3</td>
<td>9210</td>
</tr>
<tr>
<td>b</td>
<td>1.9058 ±</td>
<td>2.8452 ±</td>
</tr>
<tr>
<td></td>
<td>0.4254</td>
<td>0.7240</td>
</tr>
<tr>
<td>t_4 = 4.48</td>
<td></td>
<td>t_4 = 3.93</td>
</tr>
</tbody>
</table>

* Only 25 aneuploid-containing asci counted.
FIGURE 4.1 The effect of pFPA concentration on aneuploid induction in buff and grey-6 crosses

Aneuploid frequency ($\times 10^{-6}$)

pFPA conc. (mg/l)

x - buff
○ - grey - 6
Treatment with 1 mg/l also increased the frequency of aneuploidy, but in these cases the increase was not statistically significant.

The results show that with increasing dose there is an increased induction of aneuploidy. In order to test the statistical significance of the dose response, linear regressions were calculated and the slopes tested for significant differences from zero. The slope \( b \) of each regression line is shown in Table 4.2. Although standard errors are high the slopes of the regressions are significantly different from zero (grey-6, \( P < 0.01 \); buff \( P < 0.02 \)) and there is a dose-related response to pFPA.

Although the slopes of the regression are different for the buff and grey-6 systems, the standard errors are large and it is not possible to say that the two systems have different sensitivities to pFPA induction. There is no evidence from this data of any threshold concentration below which no induction occurred. Any threshold, if it exists, must be at lower concentrations than those examined.

Fitting a linear regression to show that there is a dose related response is a statistical device. It is not meant to imply that the response is linear. The only firm conclusion to be made from this data is that for both the buff and the grey-6 system increasing the dose does cause increased induction.
c) Is the distribution of ascus types altered? If so which class shows a disproportionate increase?

Table 4.3 gives a breakdown of the types of ascus found in the crosses whose overall aneuploid frequencies are given in Table 4.1. Heterogeneity $\chi^2$ tests show no statistically significant differences among distributions at any one dose and so the results were pooled as in Table 4.3. The distributions of ascus types found in control and treated crosses were then compared. For both buff and grey-6 crosses it was clear that pFPA had altered the distribution of ascus types. (buff 20 mg/l, $\chi^2_4 = 57.01$, $P << 0.001$; buff 10 mg/l, $\chi^2_4 = 59.12$, $P << 0.001$; grey 20 mg/l, $\chi^2_3 = 32.67$, $P << 0.001$). The differences between 10 mg/l and 20 mg/l buff crosses were not statistically significant ($\chi^2_4 = 2.79$, $P > 0.5$).

The data presented in Table 4.3 for the distribution of ascus types in treated crosses are illustrated in Figure 4.2. The ascus types are expressed as a proportion of the aneuploid-containing asci, as in the spectra in Chapter 3. The generalised spectra for spontaneous aneuploidy are shown for ease of comparison. When calculating the spectra and analysing the data the unclassified category of ascus was omitted. The purpose of the analysis was to determine whether any particular ascus type was affected by pFPA. The proportion of unclassified asci was known to be variable, according to the condition of the perithecia, and so no specific effect of pFPA would be detectable. They were therefore excluded from the analysis, and the proportions used to draw the histograms were
TABLE 4.3  Distributions of aneuploid-containing asci following treatment with p-fluorophenylalanine (10 or 20 mg/l)

<table>
<thead>
<tr>
<th>System &amp; Dose</th>
<th>Expt. No.</th>
<th>Ascus Type</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Misc.</th>
<th>Unclass.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buff 10 mg/l</td>
<td>1</td>
<td>19</td>
<td>6</td>
<td>16</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26</td>
<td>4</td>
<td>17</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>20</td>
<td>5</td>
<td>13</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>18</td>
<td>13</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>31</td>
<td>8</td>
<td>9</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Pooled</td>
<td></td>
<td>114</td>
<td>36</td>
<td>65</td>
<td>14</td>
<td>16</td>
<td>5</td>
<td></td>
<td>250</td>
</tr>
<tr>
<td>Buff 20 mg/l</td>
<td>5</td>
<td>25</td>
<td>1</td>
<td>13</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>43</td>
<td>9</td>
<td>31</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>25</td>
<td>5</td>
<td>12</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20</td>
<td>8</td>
<td>14</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Pooled</td>
<td></td>
<td>113</td>
<td>23</td>
<td>70</td>
<td>11</td>
<td>18</td>
<td>15</td>
<td></td>
<td>250</td>
</tr>
<tr>
<td>Buff controls</td>
<td>Pooled</td>
<td>290</td>
<td>25</td>
<td>51</td>
<td>70</td>
<td>26</td>
<td>38</td>
<td></td>
<td>500</td>
</tr>
<tr>
<td>Grey 20 mg/l</td>
<td>2a</td>
<td>16</td>
<td>5</td>
<td>19</td>
<td>-</td>
<td>10</td>
<td>0</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>12</td>
<td>7</td>
<td>26</td>
<td>-</td>
<td>5</td>
<td>0</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>DR</td>
<td>23</td>
<td>5</td>
<td>13</td>
<td>-</td>
<td>7</td>
<td>2</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Pooled</td>
<td></td>
<td>51</td>
<td>17</td>
<td>58</td>
<td>-</td>
<td>22</td>
<td>2</td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>Grey controls</td>
<td>Pooled</td>
<td>118</td>
<td>19</td>
<td>28</td>
<td>-</td>
<td>11</td>
<td>0</td>
<td></td>
<td>166</td>
</tr>
<tr>
<td>Grey 10 mg/l</td>
<td>DR</td>
<td>20</td>
<td>5</td>
<td>18</td>
<td>-</td>
<td>6</td>
<td>1</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>
FIGURE 4.2 (a) The distribution of ascus types in buff crosses treated with pFPA.
FIGURE 4.2 (b) The distribution of ascus types in grey-6 crosses treated with pFPA.
calculated from the total classified asci.

Examination of Figure 4.2 reveals that in all cases, in either buff or grey crosses, pFPA treatment resulted in a decreased proportion of type 1 asci, and an increased proportion of type 3 asci. The proportion of type 2 asci varied around the control level. Treated grey crosses showed a much greater proportion of type 3 asci, than did treated buff crosses. However, the control level for grey crosses is greater. In treated buff crosses type 4 asci formed a decreased proportion of the classified aneuploid-containing asci.

As the frequency of the ascus types is expressed in proportions, an increase in one type will result in a decrease in another and vice versa. The decreased proportions of type 1 and type 4 asci could be due to a lack of induction of these types, or to their being induced but type 3 asci showing a greater increase than these types. To illustrate that types 1-3 were all induced by pFPA treatment, the frequencies of individual ascus types are given in Table 4.4 and shown graphically in Figure 4.3. Linear regressions confirmed that the frequencies of ascus types 1-3 showed a dose-related response with values of b being significantly different from zero (Table 4.4). The frequency of type 4 asci did not show a dose-related response. (The value of $b = 0.1406 \pm 0.1004$, $t = 1.400, P > 0.2$). As both first and second division non-disjunction are increased by pFPA treatment, it might be expected that mitotic non-disjunction in the division preceding meiosis
TABLE 4.4 The effect of p-fluorophenylalanine concentration on the distribution of ascus types.

<table>
<thead>
<tr>
<th>pFPA dose (mg/l)</th>
<th>Numbers (and frequency x 10^-4) of each ascus type</th>
<th>Unclassified n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Buff</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>23 (3.8)</td>
<td>5 (0.8)</td>
</tr>
<tr>
<td>1</td>
<td>29 (6.3)</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>5</td>
<td>26 (9.0)</td>
<td>4 (1.4)</td>
</tr>
<tr>
<td>10</td>
<td>31 (16.7)</td>
<td>8 (4.3)</td>
</tr>
<tr>
<td>15</td>
<td>17 (8.1)</td>
<td>7 (3.3)</td>
</tr>
<tr>
<td>20</td>
<td>20 (21.7)</td>
<td>8 (8.7)</td>
</tr>
<tr>
<td>b</td>
<td>0.681±0.260</td>
<td>0.361±0.079</td>
</tr>
<tr>
<td>Grey-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>36 (5.0)</td>
<td>9 (1.3)</td>
</tr>
<tr>
<td>1*</td>
<td>14 (4.5)</td>
<td>3 (1.0)</td>
</tr>
<tr>
<td>5</td>
<td>27 (8.1)</td>
<td>3 (0.9)</td>
</tr>
<tr>
<td>10</td>
<td>20 (5.6)</td>
<td>5 (1.4)</td>
</tr>
<tr>
<td>15</td>
<td>22 (14.5)</td>
<td>7 (4.6)</td>
</tr>
<tr>
<td>20</td>
<td>23 (33.9)</td>
<td>5 (7.4)</td>
</tr>
<tr>
<td>b</td>
<td>1206±0.374</td>
<td>0.300±0.060</td>
</tr>
</tbody>
</table>

* Only 25 aneuploid-containing asci counted.
FIGURE 4.3 The effect of pFPA on the frequencies of ascus types 1-6

a) buff crosses

b) grey-6 crosses
would also be increased, and this would be one source of type 4 asci. The lack of effect of pFPA on type 4 asci may indicate that their more likely origin is through extrareplication of the appropriate linkage group prior to meiosis, than through mitotic non-disjunction. Another explanation is that pFPA only affects meiotic divisions in Sordaria. This seems unlikely as it is known to cause chromosome loss at mitosis in *Aspergillus* species (Lhoas, 1961; Morpurgo, 1961).

From Table 4.2 it is clear that the observed proportion of type 3 asci increased at high doses of pFPA. Figure 4.3 illustrates that all ascus types, except type 4 increased in frequency following pFPA treatment. It would therefore appear that the decrease in proportion of type 1 asci must be due not to lack of induction of first division non-disjunction or non-conjunction, but to a greater induction of type 3 asci. From Figure 4.3 it can be seen that the frequency of type 3 asci increases with pFPA concentration but it is not clear whether there is a relationship between dose and the proportion of aneuploid-containing asci which are type 3. These data are given in Table 4.5, where type 2 data are also presented to illustrate that the effect on type 3 asci is due to second division errors and not premature centromere division at the first meiotic division. A weighted regression was computed, using as a simple weighting the number of experiments carried out at any one concentration. The $b$ values are given in Table 4.5 and it is clear that, with increasing pFPA concentration, type 3
TABLE 4.5. The effect of p-fluorophenylalanine concentration on the proportions of type 2 and type 3 asci.

<table>
<thead>
<tr>
<th>Dose (mg/l)</th>
<th>Type 2</th>
<th>Type 3</th>
<th>Weighting (No. expts)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a) Buff</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.05</td>
<td>0.11</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>0.02</td>
<td>0.13</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0.08</td>
<td>0.16</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>0.15</td>
<td>0.26</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>0.15</td>
<td>0.29</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>0.10</td>
<td>0.29</td>
<td>5</td>
</tr>
<tr>
<td>b = 0.3400</td>
<td>b = 0.9866</td>
<td></td>
<td></td>
</tr>
<tr>
<td>± 0.1841</td>
<td>± 0.1601</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>t</em> = 1.8466</td>
<td><em>t</em> = 6.1615</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P &gt; 0.2)</td>
<td>(P &lt; 0.01)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| **b) Grey** |        |        |                      |
| 0          | 0.14   | 0.16   | 2                    |
| 1          | 0.12   | 0.28   | 1                    |
| 5          | 0.06   | 0.27   | 1                    |
| 10         | 0.10   | 0.37   | 1                    |
| 15         | 0.14   | 0.36   | 1                    |
| 20         | 0.11   | 0.39   | 3                    |
| b = -0.0384 | b = 0.9675 |
| ± 0.1447  | ± 0.2166 |
| *t* = 0.2655 | *t* = 4.4662 | |
| (P > 0.5)  | (P < 0.05) |
asci form a greater proportion of the aneuploid containing asci. There is no dose related response for type 2 asci.

The results confirm the conclusion from overall aneuploid frequencies that the effect of pFPA is not linkage group specific. The same response is found for both buff and grey-6 systems. Ascus types 1-3 are all increased in frequency, following pFPA treatment, but all types are not affected equally. The greatest effect is on type 3 asci. The difference in response in type 2 and 3 is the result expected if second division errors are increased more than premature centromere division at the first meiotic division.

In Chapter 3 it was shown that, assuming spindle overlap affects half the total asci and that all ascus types are affected equally, the observed number of type 3 asci with a 2:2:2:2 spore sequence would equal the number with a 2:2:4 sequence. This was not found and it was postulated that spindle overlap was decreased among second division non-disjunction asci. Table 4.6 gives the numbers of each spore sequence observed in crosses treated with 10 mg/l and 20 mg/l pFPA. Although the excess of the 2:2:4 sequence is not statistically significant in pFPA treated crosses, there is always a greater number of 2:2:4 than 2:2:2:2 asci. This suggests that there is for some reason, an imbalance maintained between the numbers of these two spore sequences.

As discussed in Chapter 3, the proportions of detected aneuploid-containing asci due to second division errors, can be calculated from the difference between type 2 and type 3 asci, and these values are shown in Table 4.7 for
TABLE 4.6 The numbers of 2:2:4 and 2:2:2:2 asci found among type 3 asci in p-fluorophenylalanine treated crosses.

<table>
<thead>
<tr>
<th></th>
<th>2:2:2:2</th>
<th>2:2:4</th>
<th>Total Type 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buff</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>35</td>
<td>51</td>
</tr>
<tr>
<td>10 mg/l pFPA</td>
<td>27</td>
<td>38</td>
<td>65</td>
</tr>
<tr>
<td>20 mg/l pFPA</td>
<td>27</td>
<td>43</td>
<td>70</td>
</tr>
<tr>
<td>Grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>20 mg/l pFPA</td>
<td>26</td>
<td>32</td>
<td>58</td>
</tr>
</tbody>
</table>

TABLE 4.7 The observed proportions and actual proportions of various meiotic errors in crosses treated with 20 mg/l p-fluorophenylalanine.

<table>
<thead>
<tr>
<th>Error</th>
<th>Observed proportions</th>
<th>Actual Proportions</th>
</tr>
</thead>
<tbody>
<tr>
<td>First division non-disjunction and non-conjunction</td>
<td>0.48</td>
<td>0.34</td>
</tr>
<tr>
<td>Premature centro-mere division</td>
<td>0.20</td>
<td>0.23</td>
</tr>
<tr>
<td>Second division errors</td>
<td>0.15</td>
<td>0.27</td>
</tr>
<tr>
<td>Extra replication or mitotic non-disjunction</td>
<td>0.30</td>
<td>-</td>
</tr>
</tbody>
</table>
crosses treated with 20 mg/l pFPA. These are proportions of detected aneuploid-containing asci. In Chapter 3 it was shown that correction factors could be applied to allow for the fact that the efficiency of detection of different meiotic errors is not constant. The correction factors given in Chapter 3 have been applied to the buff and grey-6 data. Table 4.7 also shows the occurrence of each meiotic error as a proportion of the estimated total of these errors obtained by using the correction factors. It is clear from these figures that following pFPA treatment the majority of meiotic errors resulting in aneuploidy are those arising at the second meiotic division. This conclusion applies to both linkage group II and linkage group IV.

d) Recombination and pFPA induction

As the detection of second division non-disjunction depends on crossing-over having occurred between the spore colour mutant loci and the centromere, one explanation for the increased proportions of type 3 asci could be that pFPA was causing a general increase in recombination frequency. If this was true, the frequency of 2+:6- asci would be expected to increase following treatment of grey-6 crosses with pFPA. Figure 4.4 shows the frequencies of 2+:6- asci observed in the grey-6 dose response experiment. Fitting a linear regression gave a value of $b = 0.0067 \pm 0.0205$, with $t_4 = 0.322, P >> 0.05$. There is no evidence to suggest that the increased levels of type 3 asci were caused by increased recombination.
FIGURE 4.4 The effect of pFPA on the frequency of 2×:6- asc in RW 25 × C31 (gray-6) crosses
e) **Time of application and induction by pFPA**

As type 1 and type 2 asci arise through first division errors, and type 4 asci through premeiotic errors, it is possible that the large increase in type 3 asci is due to the second meiotic division being more sensitive to pFPA, resulting in increased levels of second division non-disjunction. The greater effect at this division could be due to a lag in incorporation of pFPA. The asynchronous nature of *Sordaria* crosses means that at any one time many stages of meiosis are open to attack, and so increases would be noted in all meiotic errors. However, at any one time after fertilization there may be a predominance of a particular stage. If pFPA uptake was such that the highest intracellular concentration was achieved when the predominant stage was the second meiotic division, this would explain the greater sensitivity of this division.

To investigate this possibility pFPA was applied to the female plates at various times prior to fertilization. If the above hypothesis was true, earlier treatment should result in an increased proportion of first division errors, and hence an increased proportion of type 1 and type 2 asci, compared to the levels observed following treatment at the time of fertilization. Previous work by Bond (unpublished) had shown no increase in the proportion of first division errors following treatment two or four hours prior to fertilization. Treatment was therefore carried out at earlier times, twelve, twenty-four and forty-eight hours before fertilization. The results obtained are given in
Table 4.8 and the spectra of ascus types illustrated in Figure 4.5. The first observation is that early treatment results in a lower induction of aneuploidy, than treatment at fertilization. Possibly more of the pFPA is incorporated into proteins not involved in meiosis, if applied prior to fertilization, thus effectively reducing the concentration of pFPA to which meiotically dividing chromosomes are exposed.

From the data it appeared that earlier application time did not affect the spectrum of ascus types and this was confirmed by 2xn heterogeneity $\chi^2$ tests on the spectra. No statistically significant differences were found in pFPA treated crosses. Comparing the distributions of ascus types at time zero with those at 12, 24 and 48 hours gave $\chi^2$ values of 1.305 ($P >> 0.5$), 6.31 ($P > 0.1$) and 3.13 ($P > 0.5$) respectively. These data suggest that a lag in pFPA incorporation is not the explanation for the apparent high sensitivity of the second meiotic division to pFPA.

3. The action of pFPA at meiosis

The mode of action of pFPA to induce meiotic aneuploidy is unclear. It is known that pFPA is incorporated into many proteins in place of phenylalanine (Cohen and Gros, 1960). This can result in normally functioning protein, for example, aldolase and glyceraldehyde-3-phosphate dehydrogenase (Westhead and Boyer, 1961), or can have severe effects. Flagella of *Salmonella typhimurium* regenerates in the presence of pFPA but are non-functional (Kerridge, 1960). Another action of pFPA is to reduce protein synthesis. It is, therefore, possible that the effects of
TABLE 4.8. The effect of application time on the numbers (and frequencies x 10^{-4}) of ascus types induced by p-fluorophenylalanine.

<table>
<thead>
<tr>
<th>Ascus type</th>
<th>Control</th>
<th>Time of application prior to fertilization/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>28 (6.9)</td>
<td>43 (39.2)</td>
</tr>
<tr>
<td>2</td>
<td>0 (-)</td>
<td>9 (8.2)</td>
</tr>
<tr>
<td>3</td>
<td>5 (1.2)</td>
<td>31 (28.3)</td>
</tr>
<tr>
<td>4</td>
<td>5 (1.2)</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>7 (1.7)</td>
<td>8 (7.3)</td>
</tr>
<tr>
<td>Unclassified</td>
<td>5 (1.2)</td>
<td>8 (7.3)</td>
</tr>
<tr>
<td>Total</td>
<td>50 (12.4)</td>
<td>100 (91.2)</td>
</tr>
<tr>
<td>Total Asci screened</td>
<td>40280</td>
<td>10960</td>
</tr>
</tbody>
</table>
FIGURE 4.5 The distribution of ascus types following pFPA treatment at various times prior to fertilization (pFPA concentration 20 mg/l)

proportion of classified asci

ASCUS TYPE

Treatment time 0hr - 12hr - 24hr - 48hr

all times: n=100
pFPA are the result of its incorporation into proteins or of the reduction in protein synthesis. Sisken and Wilkes (1967) demonstrated that the effect of pFPA on mitosis was due to its incorporation into proteins.

It seems likely that the action of pFPA is to prevent the normal functioning of a 'meiosis-related' protein or proteins, resulting in abnormal chromosome segregation. Three of the more obvious groups of proteins which could come into this category are: those involved in recombination and pairing of homologues; those involved in the meiotic spindle and those involved in the maintenance of chromatid attachment at the centromere until anaphase II.

Ascus analysis of pFPA-induced aneuploidy in Sordaria has revealed that, although pFPA causes some induction of first division errors, the most sensitive stage of meiosis is the second meiotic division. This suggests that the major effect of pFPA is not achieved through altered recombination. Confirmation of this point comes from the observation that pFPA does not affect recombination in Sordaria. Griffiths and Delange (1977) have also reported no change in recombination frequency following pFPA treatment, using Neurospora crassa. The lack of effect on recombination eliminates the possibility that pFPA is acting to reduce chromosome pairing and hence increase non-conjunction. It also rules out any increase in first division non-disjunction arising through increased recombination.

The Sordaria system has revealed that three main types
of meiotic error are induced by pFPA and these are first and second division non-disjunction and premature centromere division. Increased non-disjunction may arise through altered meiotic spindles but premature centromere division requires a different explanation, such as lack of control over the separation of chromatids. It could be that pFPA acts on chromosome segregation in more than one way. This would not be surprising if it is incorporated into several proteins.

pFPA and the spindle. Reports of the action of pFPA on mitosis have shown that, at least in this division, the spindle is affected. Biesele and Jacquez (1954) noted multipolar spindles when treating mouse sarcoma culture cells with pFPA. Sisken and Wilkes (1967) found no change in the general appearance of spindles in pFPA treated human amnion culture cells, but did notice that they decreased in size. Sisken et al. (1972) also found this decrease in size, but the spindle morphology was altered with treated spindles being less dense than controls, and often having divided poles, where spindle fibres converged on more than one centre.

Other work which supports the hypothesis that pFPA interacts with the spindle is that of Raybin and Flavin (1975) and Rodriguez and Borisy (1978, 1979). Raybin and Flavin demonstrated the existence of an enzyme which added tyrosine to the C-terminal of brain α-tubulin (also a major spindle component) in a post-translational modification. Not all α-tubulin was modified in this way. Tyrosylation
did not affect the ability of the tubulin to polymerise in vitro, but may be important to the functioning of the tubulin in vivo. Rodriguez and Borisy (1978) found that a small proportion of α-tubulin had phenylalanine bound at the C-terminal, in place of tyrosine, the ratio of phenylalanine to tyrosine being proportional to their cellular concentrations. Further work (Rodriguez and Borisy, 1979) showed that phenylalanine and tyrosine compete for the C-terminal binding site, and increasing concentrations of phenylalanine cause tyrosine to be replaced. Treatment with pFPA may result in pFPA replacing tyrosine at this site, perhaps altering the functioning of the spindle.

pFPA and the duration of mitosis. A second effect of pFPA at mitosis is to increase the duration of the mitotic process (Biesele and Jacquez, 1954). Sisken and Wilkes (1967) showed this effect to be more specific - the increase they observed was due to prolonged metaphases, with little or no effect on the rest of the mitotic cycle. Sisken and Iwasaki (1969) examined the action of other amino acid analogues, and found that only phenylalanine analogues gave this specific effect on metaphase. The most pronounced increase in duration was obtained using pFPA. They also observed that, while pFPA prolonged the first metaphase following treatment, the major effect was on the second division, when metaphase was often completely blocked. The higher the concentration of pFPA, the fewer the cells which passed the second division after treatment.
Wheatley and Henderson (1975) reported that pFPA increased the duration of metaphase in HeLa cells already committed to mitosis, but blocked entry into mitosis for other cells, resulting in a decreased mitotic index. This conflicts with the results of Sisken et al. (1972) who found an increase in mitotic index following pFPA treatment of HeLa cells. These differences in response may be due to the widely differing concentrations used by these workers. Wheatley and Henderson (1975) were using 0.2 mM pFPA and the effect was reversible while Sisken et al. (1972) used 10 mM pFPA and increasing treatment times caused a decrease in reversibility of the response.

Despite these differences it is clear that at mitosis pFPA has two main effects - the spindle morphology is altered and, at least in some cells the duration of metaphase is increased. These two effects may, or may not, be the result of the same basic defect. The fact that the mitotic spindle is altered by pFPA gives some support to the suggestion that pFPA induces aneuploidy by altering the meiotic spindle, but it is not conclusive evidence.

It is interesting that Sisken and Iwasaki (1969) noted that the major effect of pFPA was on the second mitotic division after treatment and that in Sordaria meiotic errors originating at the second division were increased more than those due to defects at the first division. It may be that there is a pool of meiotic or mitotic proteins and that it is not until these are exhausted that proteins with incorporated pFPA have an effect.
The slight increase in premature centromere division at the first meiotic division may arise through pFPA uncoupling the processes of chromosome movement and chromatid separation, for example, as a result of prolonged metaphase. This may also contribute to the increase in second division errors, as these can also arise through premature separation of chromatids.

If the action of pFPA required multiple events, for example to damage the spindle, then a threshold concentration might be expected to be observed, below which no effect was noticed. However detecting the presence or absence of a threshold is always beset with problems relating to the size of the experiment. Obviously small effects require large experiments to obtain statistical significance. To distinguish a genuine lack of effect (threshold) because a large enough proportion of subunits remain unaltered from a small effect (only a slightly increased chance of affecting enough subunits) can be very difficult. The lack of statistical significance of the increase in aneuploid frequency following treatment with 1 mg/1 pFPA does not automatically imply that this is a threshold concentration.

4. Summary

It has been demonstrated that pFPA induces meiotic aneuploidy in Sordaria, affecting both first and second division errors but not premeiotic errors. The major effect is on the second meiotic division. Similar results
have been obtained for both linkage group II and linkage group IV.

Some suggestions have been made to explain the induction of meiotic aneuploidy by pFPA, based on the observations in *Sordaria* and reports of the effects of pFPA on mitosis. pFPA could induce aneuploidy by altering the spindle, so causing increased non-disjunction of chromosomes and chromatids. There is no evidence that it causes increased non-conjunction of chromosomes. Prolonged metaphases may also contribute to aneuploidy, perhaps increasing the probability of premature centromere division. These effects could be the result of the same or different defects arising through incorporation of pFPA into proteins, either during translation or by post-translational modification. Care has to be taken in extrapolating from mitosis to meiosis and from mammalian cell culture to fungi. As pFPA has not given any increase in aneuploidy in mice (Brook, 1983) it may be that it acts differently in mammals than in fungi. It is also possible that the pFPA administered to mice may have been metabolized before reaching the germ cells. Another possibility is that part of the action of pFPA is on the nuclear membrane in fungi. Unlike in mammals, this remains intact during cell division in fungi and interference with the membrane may disrupt chromosome segregation. Having established that pFPA did induce aneuploidy in both buff and grey systems it was possible to use this compound as a positive control in other chemical treatment experiments.
1. Introduction

It has been established that spontaneous aneuploidy could be detected for two linkage groups of *Sordaria brevicollis* and that the systems were sensitive to induction by pFPA. To assess the usefulness of *Sordaria* ascus analysis as a screening system for aneuploid inducers it was necessary to investigate how it responded to known aneuploid inducers, other than pFPA. In a limited time it was not possible to study a large number of compounds. It was therefore decided to investigate the effects of one group of compounds.

The class chosen was spindle inhibitors. These are obvious candidates as aneuploid inducers since the spindle is involved in chromosome separation and meiotic mutants have been described which affect the spindle and result in aneuploidy (Chapter 1). There were several reasons for studying spindle inhibitors.

a) By definition their site of action in the cell is known.

b) If no other cellular component is affected, they are unlikely to have a genetic consequence other than aneuploidy or polyploidy. This is the class of agent which aneuploid testing systems are intended to detect.

c) The main protein component of the meiotic spindle is tubulin. There is evidence that lower and higher eukaryotic tubulin may be different, for example in binding affinity for some spindle inhibitors (see
below). This indicates that a fungal system may react differently to aneuploid induction by spindle inhibitors than would a mammalian system.

d) Some spindle inhibitors have been reported as aneuploid inducers in mammals. It would therefore be possible to investigate the response of the *Sordaria* system to such chemicals.

e) Spindle inhibitors have been studied in other fungal test systems and so the response of the *Sordaria* system could be compared to these.

Some evidence for variation in tubulin between lower and higher eukaryotes comes from biochemical and immunological comparisons of different tubulins. Paramecium α and β tubulin are different from those of the vertebrates (pig, mouse and chicken) in electrophoretic mobilities, peptide maps and immunological reactions (Adoutte et al., 1982). There are also differences between *Physarum* and sheep α tubulin (Clayton et al., 1980), and tubulin from *Chlamydomonas* flagella shows differences to brain tubulin (Olmsted et al., 1971). However amoebal and brain tubulins co-polymerise in experimental conditions, indicating that there are also similarities between lower and higher eukaryotic tubulins (Roobol et al., 1980).

Spindle inhibitors or spindle poisons, include a large number of chemicals of which only some could be selected for study. The compounds chosen were colchicine, vinblastine, benomyl and methyl-2-benzimidazole carbamate (MBC), for reasons outlined below.
2. **Materials and Methods**

Treatment of crosses was carried out as for pFPA, unless otherwise stated. Benomyl and MBC are not water soluble. Ethanol was rejected as a solvent because *Sordaria* does not cross in the presence of ethanol (Bond, unpublished). There is also some evidence that ethanol induces aneuploidy in fungi (Harsanyi et al., 1977). Dimethylsulfoxide (DMSO) was used as a solvent at a final concentration of 0.5% (V/V). DMSO is widely used as an inactive solvent in mutagenicity testing and had been used as a solvent in aneuploid testing in *Aspergillus nidulans* and had shown no effects on aneuploidy (Bignami et al., 1977). DMSO had previously been shown to be non-toxic to *Sordaria* at the concentration used (Bond, unpublished).

Colchicine was obtained from Sigma (C9754) and vinblastine was administered as the water soluble vinblastin sulphate, also obtained from Sigma (V1377). Benomyl and MBC were the gift of Dupont (U.K.) Limited, Hemel Hempstead, England and were of analytical standard 99+%

For each compound toxicity tests were carried out with a range of concentrations. Normally the concentrations chosen for use in aneuploid testing were those up to the concentration at which crosses were beginning to show decreased fertility.

All aneuploid induction tests were carried out using the **buff** detection system.
3. The effects of spindle inhibitors on aneuploidy

a) Colchicine

Colchicine was chosen for testing because this compound was known to induce errors in chromosome segregation at mitosis and meiosis in mammals, and also because the sensitivity to colchicine differed in lower and higher eukaryotes. Chromosome segregation errors induced by colchicine or its derivative colcemid were reported in mammals both in vivo (Edwards, 1954, 1958; Sugawara and Mikamo, 1980) and in vitro (Cox and Puck, 1969; Kato and Yoshida, 1970, 1971; Cox, 1973). Induction of sex chromosomal aneuploidy was reported in Drosophila (Traut and Scheid, 1974; Ramel and Magnusson, 1979; Held, 1982). All these were at very low concentrations, for example 3.75 x 10^{-8} M (Cox and Puck, 1969) and 2.5 x 10^{-6} M (Traut and Scheid, 1974). However in lower eukaryotes, 5 x 10^{-3} M colchicine produced no effect on Penicillium notatum (Sansome and Bannon, 1946) and concentrations greater than 1.5 x 10^{-3} M were required to prevent microtubule assembly in Chlamydomonas reinhardtii (Flavin and Slaughter, 1974). Even higher concentrations of 1.0 x 10^{-2} - 2.5 x 10^{-2} M were found necessary to block cell division in Dictyostelium discoideum and Polysphondylium pallidum (Cappucinelli and Ashworth, 1976; Williams, 1980; Zada-Hames, 1977). In Saccharomyces cerevisiae colchicine gave no effect and 10^{-3} to 10^{-2} M colcemid affected cell division, but only in restricted growth conditions (Haber et al., 1972). Colchicine binds irreversibly to a site in mouse brain α-tubulin and reversibly to sites on both α and β tubulin (Schmitt and Atlas, 1976). Further binding studies
have revealed that lower eukaryotic tubulin has a much lower binding affinity for colchicine than higher eukaryotic tubulin. Polymerisation of brain tubulin is strongly inhibited by $10^{-4}$ M colchicine but tubulin from *Physarum polycephalum* is unaffected (Roobol et al., 1980). Similar low affinities have been found in *Tetrahymena* (Maekawa and Sakai, 1978) and *Aspergillus* (Davidse and Flach, 1977).

The findings that lower eukaryotes shared low sensitivity to growth inhibition by colchicine and that their tubulin had low binding affinities for colchicine suggested that although aneuploidy was induced in mammals, a fungal system would fail to detect aneuploid induction by colchicine. In order to test this idea *Sordaria* crosses were treated with colchicine.

**Treatment concentrations**

The *in vivo* effects of colchicine in lower eukaryotes had been tested in mitotic systems, and it was possible that meiosis may have been more sensitive to colchicine. Also aneuploid induction was expected to occur at concentrations below those necessary to cause complete arrest of microtubule function. Concentrations tested were $5 \times 10^{-5}$ to $2 \times 10^{-3}$ M colchicine. None of these conditions caused any reduction in the fertility of crosses.

**Analysis of the results of colchicine treatment**

Aneuploid counts were carried out using the material from crosses treated with $2 \times 10^{-3}$ M colchicine. The results obtained are given in Table 5.1. It is clear
### TABLE 5.1. Aneuploid frequencies following treatment with colchicine.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Asci Counted</th>
<th>Aneuploid Frequencies x 10^-4</th>
<th>Ascus Types</th>
<th>Unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>H2O</td>
<td>65840</td>
<td>7.6</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td>pFPA 10mg/l</td>
<td>21810</td>
<td>22.9</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Colchicine 2 x 10^-3M</td>
<td>70560</td>
<td>7.1</td>
<td>29</td>
<td>1</td>
</tr>
</tbody>
</table>

### TABLE 5.2. Aneuploid frequencies following treatment with vinblastine sulphate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Asci Counted</th>
<th>Aneuploid Frequencies x 10^-4</th>
<th>Ascus Types</th>
<th>Unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>H2O</td>
<td>70250</td>
<td>.7.2</td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td>pFPA 20mg/l</td>
<td>5850</td>
<td>85.5</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>Vinblastine sulphate 1.1 x 10^-4M</td>
<td>65850</td>
<td>7.6</td>
<td>35</td>
<td>1</td>
</tr>
</tbody>
</table>
that the frequency of total aneuploidy and the distribution of ascus types was not changed with colchicine treatment. The pFPA control showed that the system was capable of detecting induced aneuploidy.

Colchicine at $2 \times 10^{-3} \text{M}$ is almost $10^5$ times the concentration which induced mitotic effects in mammals and no response was detected in *Sordaria*. This lack of effect was not unexpected considering the high tolerance to colchicine observed in other lower eukaryotes. Repeat crosses and a different method of treatment were also tried. Crosses were set up on CMA but the membranes were transferred to colchicine-containing media on days 1 and 2 after inoculation. On fertilization the mycelium had therefore been growing on colchicine for two or three days. A quick scan of material from these crosses showed no obvious changes in aneuploid frequency and so no more detailed counts were made. These experiments demonstrated that, like mitosis in other lower eukaryotes, mitosis and meiosis in *Sordaria* were much less sensitive to colchicine than mammals.

Östergren and Levan (1943) and Östergren (1951) showed a connection between 'c' mitotic activity and water solubility of organic compounds, and this was further discussed by Onfelt and Ramel (1979). There was a correlation between solubility in water and the threshold concentration at which "c" mitosis was detected. The lower the solubility, the lower the threshold of action. However, some compounds did not fit this relationship and had a lower threshold than would be expected from their water solubility. Compounds
which fall into this category included colchicine and various mercury compounds. This was used to argue that spindle disturbances (c-mitosis) could be induced by many compounds, if given in a certain dose related to their water solubility, but that compounds with a more specific effect on microtubules would have a much lower threshold of action than expected.

All these conclusions were based on the effect of these compounds on 'c' mitosis in Allium. Figure 5.1 shows the graph produced by Onfelt and Ramel (1979) and Östergren (1951). The open circles indicate compounds whose effect was found at lower concentrations than predicted from their solubility. The concentrations of colchicine necessary to induce mitotic arrest in lower eukaryotes place it among the main group of points, suggesting that in these organisms colchicine has an unspecific action. Concentrations greater than $2 \times 10^{-3}$ M are within the range of concentrations when compounds with the solubility of colchicine would be expected to produce a non-specific effect. Rather than continuing to apply increasing concentrations of colchicine until reduced fertility or induced aneuploidy was noted it was decided to concentrate on other possible inducers.

b) Vinblastine

Vinblastine is another compound known to interact with mammalian tubulin. Microtubular crystals were formed in L-strain fibroblasts and human leucocytes following treatment with $10^{-5}$ to $4 \times 10^{-4}$ M vinblastine (Bensch and
Figure 5.1 Illustration of the correlation between the water solubility of a number of different chemical compounds and the minimum concentration necessary for the induction of mitotic arrest (closed circles).

Some substances induce arrest at much lower concentrations than predicted from their water solubility (open circles): (1) Colchicine; (2) methyl mercury dicyandiamide; (3) methyl mercury hydroxide; (4) phenyl mercury hydroxide; (5) mercury chloride; (6) trimethyl tin chloride; (7) tributyl tin chloride; (8) diethyl lead chloride; (9) triethyl lead chloride; (10) trimethyl lead chloride; (11) lead nitrate (from Östergren (1951) and Ramel and Magnusson (1979)).
Malawista, 1969). In hamster cells $7.5 \times 10^{-8}$ M vinblastine produced 50% mitotic arrest (Wilson et al., 1974). The cell division spindles of oocytes in the annelid Pectinaria gouldi were disrupted by $10^{-5}$ M vinblastine (Malawista et al., 1968). Regeneration of flagella in Chlamydomonas reinhardtii was blocked by $10^{-4}$ M vinblastine sulphate (Flavin and Slaughter, 1974) and $10^{-3}$ M vinblastine inhibited microtubule function in Dictyostelium discoideum (Cappucinelli and Ashworth, 1976) but Heath (1975) found no effects of vinblastine on the fungus Saprolegnia ferax. Bryan (1972) and Owellen et al. (1972) demonstrated binding of vinblastine to porcine brain tubulin. Previous work by Wilson et al. (1970) and Olmsted et al. (1970) had shown that colchicine and vinblastine did not compete for a site of action and it was therefore concluded that they must have different tubulin binding sites. Vinblastine produced various configurations of tubulin aggregates when neurotubulin was treated (Warfield and Bouck, 1974) and the same configurations were observed using amoebal tubulin (Roobol et al., 1980). Because vinblastine acts on tubulin it was a potential aneuploid inducer. There was some indication that lower eukaryotes may be less sensitive than mammals from the work of Heath (1975) and the higher concentrations required to affect other lower eukaryotes compared to hamster cells. The fact that colchicine and vinblastine have different tubulin binding sites suggested that the response of Sordaria to vinblastine could be different than that to colchicine, although Griffiths (1979) reported no
significant increase following treatment of *Neurospora crassa* with an unspecified concentration of vinblastin sulphate.

**Treatment concentrations**

The concentration of vinblastin sulphate which affected lower eukaryotes was about $10^{-4}$ M. As concentrations inducing aneuploidy would be expected to be below those causing complete blockage of microtubule function, concentrations tested were $1.1 \times 10^{-6}$ M to $1.1 \times 10^{-4}$ M vinblastin sulphate. Reduced fertility of crosses was observed in $5.5 \times 10^{-5}$ M and $1.1 \times 10^{-4}$ M treatments.

**Analysis of the results of vinblastin sulphate treatment**

Aneuploid counts were made from crosses treated with $1.1 \times 10^{-4}$ M vinblastin sulphate and the results obtained are given in Table 5.2. As with colchicine no induction of aneuploidy was observed and the distribution of ascus types appeared unchanged. The pFPA control again demonstrated that the system was capable of induction. It is clear that despite the fact that vinblastin sulphate reduced cross fertility there was no sign of any increase in the frequency of aneuploidy. This may indicate that although vinblastine binds to tubulin it does not induce abnormal chromosome segregation. Another possibility is that the concentration required to produce aneuploidy is very close to that which kills the cell, that is the threshold for aneuploid induction is very close to the toxicity threshold. If this is true then in most cells where
the concentration reached sufficiently high levels to affect the segregation of chromosomes it may also be sufficient to cause cell death. It may be significant that vinblastine causes crystal formation within cells (Bensch and Malawista, 1969). This could interfere with cellular functions other than division, leading to cell death. The result may also be due to insensitivity of fungal testing systems, as Heath (1975) found *Saprolegnia ferax* to be tolerant of vinblastine.

To demonstrate that vinblastine did not induce aneuploidy but did bind to tubulin it would be necessary to extract fungal tubulin and examine the effects of vinblastine on tubulin polymerisation. This would indicate whether the fungal testing systems detected no aneuploid induction because of lack of interaction of tubulin with vinblastine or because vinblastine did not have a detectable effect on aneuploidy in fungi.

c) **Benomyl and methyl-2-benzimidazole carbamate**

Benomyl or methyl-1-(butylcarbamoyl)-2-benzimidazole and its metabolic derivative methyl-2-benzimidazole carbamate (MBC) are widely used fungicides which act by binding to tubulin. Davidse and Flach (1977) showed MBC was a competitive inhibitor of colchicine but not vinblastine using tubulin from *Aspergillus nidulans*. This indicates that colchicine and MBC have the same or very similar binding sites on tubulin, such that binding of one blocks access to tubulin for the other compound, at least for fungal tubulin.
Quinlan et al. (1981) reported brain tubulin was less sensitive to MBC than tubulin from Physarum polycephalum. However MBC and benomyl have been found to affect mitosis in mammals and fungi. Styles and Garner (1974) showed mitotic arrest was induced by MBC and benomyl in various mammalian cell culture lines and in vivo in rats, and Seiler (1976) reported unequal distribution of chromatin at mitosis in Chinese hamster and mouse cells following MBC treatment. This could be indicative of aneuploid induction. Benlate (50% benomyl) caused mitotic arrest in Polysphondylium pallidum when applied as 100μg/ml (Williams, 1980). MBC and benomyl have proved positive in mitotic aneuploidy induction tests using Aspergillus nidulans (Hastie, 1970; Kappas et al., 1974; Kappas, 1978; Bignami et al. 1977; Morpurgo, et al., 1979).

The effects of MBC and benomyl on the Sordaria aneuploid detection system were studied to allow comparison of the sensitivity of the Sordaria system with other fungal systems. These compounds were also interesting because they bound to tubulin at a similar site to colchicine.

**Treatment concentrations**

Concentrations of these compounds which affected mitosis in Aspergillus nidulans but did not cause complete mitotic arrest were 1.75 μM benomyl and 2.5 μM MBC dissolved in ethanol (Kappas et al., 1974). 1.5 μM benomyl dissolved in DMSO caused increased mitotic aneuploidy in Aspergillus (Bignami et al., 1977). Toxicity tests with Sordaria
showed that perithecia were formed at treatment concentrations up to 2.5 μM benomyl and 5 μM MBC, although at 5 μM crosses often failed completely. Crosses to detect aneuploid induction were therefore treated with 0.1 - 2.5 μM benomyl and 0.1 - 5.0 μM MBC in the hope of obtaining dose response data.

Analysis of the results of benomyl and MBC treatment

Overall aneuploid frequencies

Table 5.3(a) shows the results obtained following treatment of crosses with 2.5 μM and 5.0 μM MBC and 2.5 μM benomyl. Comparing the water controls and MBC results it appeared that MBC was causing a slight increase in aneuploid frequency and 't' tests confirmed that the differences between treatments were statistically significant. Benomyl gave similar results to MBC. The 't' values shown in Table 5.3(b) were obtained by a simple 't' test when only one result was available or by a test of paired differences when more results were available for any one treatment concentration.

However, when comparing the MBC and benomyl results to those obtained in the solvent control there were no significant differences in aneuploid induction. Neither were there any statistically significant differences between water and DMSO controls, although DMSO treatment always resulted in a slightly inflated aneuploid frequency. The combination of MBC plus DMSO generally gave a higher frequency again, although this was not true in experiment 8. The pFPA controls demonstrated that the crosses were capable of showing large increases in the frequency of aneuploidy.
TABLE 5.3. The effect of treatment with benomyl and MBC on aneuploid frequency.

a) Results

<table>
<thead>
<tr>
<th>Expt.No.</th>
<th>H₂O Control</th>
<th>0.5% DMSO</th>
<th>2.5 μM Benomyl</th>
<th>2.5 μM MBC</th>
<th>5.0 μM MBC</th>
<th>pFPA 10mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency x 10⁻⁴</td>
<td>7.3</td>
<td>8.2</td>
<td>- 10.3</td>
<td>10.7</td>
<td>21.5</td>
<td></td>
</tr>
<tr>
<td>Total asc. screened</td>
<td>68450</td>
<td>60710</td>
<td>48740</td>
<td>46610</td>
<td>23260</td>
<td></td>
</tr>
<tr>
<td>Frequency x 10⁻⁴</td>
<td>7.6</td>
<td>10.8</td>
<td>11.7</td>
<td>12.4</td>
<td>- 22.9</td>
<td></td>
</tr>
<tr>
<td>Total asc. screened</td>
<td>65840</td>
<td>46170</td>
<td>42770</td>
<td>40280</td>
<td>21810</td>
<td></td>
</tr>
<tr>
<td>Frequency x 10⁻⁴</td>
<td>5.6</td>
<td>9.3</td>
<td>- 8.9</td>
<td>-</td>
<td>19.4</td>
<td></td>
</tr>
<tr>
<td>Total asc. screened</td>
<td>89610</td>
<td>53820</td>
<td>56280</td>
<td>25750</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b) Statistical Tests

<table>
<thead>
<tr>
<th>Test</th>
<th>t value</th>
<th>d.f.</th>
<th>P</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O vs. MBC 2.5 μM</td>
<td>6.68</td>
<td>2</td>
<td>&lt;0.02</td>
<td>YES</td>
</tr>
<tr>
<td>DMSO vs. MBC 2.5 μM</td>
<td>1.44</td>
<td>2</td>
<td>&gt;0.2</td>
<td>NO</td>
</tr>
<tr>
<td>H₂O vs. MBC 5.0 μM</td>
<td>1.85</td>
<td>∞</td>
<td>&gt;0.05</td>
<td>NO</td>
</tr>
<tr>
<td>DMSO vs. MBC 5.0 μM</td>
<td>1.31</td>
<td>∞</td>
<td>&gt;0.1</td>
<td>NO</td>
</tr>
<tr>
<td>H₂O vs. Benomyl 2.5 μM</td>
<td>2.07</td>
<td>∞</td>
<td>&lt;0.05</td>
<td>YES</td>
</tr>
<tr>
<td>DMSO vs. Benomyl 2.5 μM</td>
<td>1.45</td>
<td>∞</td>
<td>&gt;0.1</td>
<td>NO</td>
</tr>
<tr>
<td>H₂O vs. DMSO</td>
<td>2.62</td>
<td>2</td>
<td>&gt;0.1</td>
<td>NO</td>
</tr>
</tbody>
</table>
Both MBC and benomyl were biologically active as judged by the effects on cross development and maturation. It was, therefore, surprising to find no statistically significant increase in aneuploid levels over those observed in the solvent control. The solvent appeared to be having a greater effect than the test compounds, although not statistically significant. The increases which were significant appeared due to a combined effect of solvent and solute.

Analysis of the distribution of ascus types

Table 5.4 shows the distribution of ascus types observed in treated crosses. Using the pooled totals for each treatment 2 x n heterogeneity $\chi^2$ tests showed significant differences in the distribution of ascus types when comparing MBC 2.5 $\mu$M results with DMSO or water results ($\chi^2_4 = 11.07, P \approx 0.025$, $\chi^2_4 = 11.5, P = 0.025$ respectively). There were no significant differences in the DMSO and water results ($\chi^2_4 = 5.25, P > 0.1$). From the breakdown of the data it is clear that the heterogeneity is mainly due to a large number of type 1 asci in some MBC 2.5 $\mu$M crosses. As this trend was not observed in all MBC 2.5 $\mu$M crosses nor in the MBC 5.0 $\mu$M or benomyl 2.5 $\mu$M crosses it is probable that the heterogeneity has no biological significance.

The effect of application time of MBC

Despite the fact that the solvent, DMSO, appeared to be causing slight increases in aneuploidy, benomyl and MBC would still have been expected to increase aneuploidy above
TABLE 5.4  The distribution of ascus types in MBC and benomyl treated crosses.

<table>
<thead>
<tr>
<th>Treatment (and experiment no.)</th>
<th>Number (and proportion of classified asci) of each ascus type</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>MBC 2.5µM</td>
<td>28 (0.64)</td>
<td>0 (-)</td>
</tr>
<tr>
<td></td>
<td>39 (0.83)</td>
<td>0 (-)</td>
</tr>
<tr>
<td></td>
<td>33 (0.69)</td>
<td>2 (0.04)</td>
</tr>
<tr>
<td>Pooled</td>
<td>100 (0.72)</td>
<td>2 (0.01)</td>
</tr>
<tr>
<td>MBC 5.0µM</td>
<td>28 (0.61)</td>
<td>4 (0.09)</td>
</tr>
<tr>
<td>Benomyl 2.5µM</td>
<td>28 (0.60)</td>
<td>3 (0.06)</td>
</tr>
<tr>
<td>DMSO 0.5%</td>
<td>28 (0.60)</td>
<td>4 (0.09)</td>
</tr>
<tr>
<td></td>
<td>36 (0.78)</td>
<td>0 (-)</td>
</tr>
<tr>
<td></td>
<td>30 (0.67)</td>
<td>1 (0.02)</td>
</tr>
<tr>
<td></td>
<td>32 (0.65)</td>
<td>1 (0.02)</td>
</tr>
<tr>
<td>Pooled</td>
<td>126 (0.67)</td>
<td>6 (0.03)</td>
</tr>
<tr>
<td>Standard H₂O</td>
<td>290 (0.63)</td>
<td>25 (0.05)</td>
</tr>
</tbody>
</table>

TABLE 5.5.  The effect of earlier application on aneuploid induction by MBC

<table>
<thead>
<tr>
<th>Time prior to fertilization</th>
<th>0 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aneuploid Frequency 10⁻⁴</td>
<td>Total Asci Screened</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O Control</td>
<td>5.6</td>
<td>89610</td>
</tr>
<tr>
<td>DMSO 0.5%</td>
<td>9.3</td>
<td>53820</td>
</tr>
<tr>
<td>MBC 2.5 µM</td>
<td>8.9</td>
<td>56280</td>
</tr>
<tr>
<td>pFPA 10mg/1</td>
<td>19.4</td>
<td>25750</td>
</tr>
</tbody>
</table>
any effect of the solvent. This was obviously not the case. As both compounds were toxic to the fungus, the solvent was not neutralising their biological activity. One possible cause of lack of effect could have been the treatment procedure. Perhaps application at fertilization did not allow sufficient time to elapse, prior to meiosis, for the MBC or benomyl to cause their effect. Earlier application of MBC was therefore tried. The results are shown in Table 5.5. In these experiments there were no significant differences between crosses treated 48 hours prior to fertilization and at fertilization. In each case there was no increase in aneuploid frequency in MBC treated crosses compared to the solvent control. The lack of effect of MBC did not therefore seem to be due to MBC not being applied sufficiently early before fertilization. For either time of treatment the water control had a significantly lower aneuploid frequency than any other cross, for example water versus DMSO at 48 hr prior to fertilization $t_{0} = 2.684, P < 0.01$. The water control was lower than any other spontaneous level of aneuploidy (Chapter 3). It is not clear whether the difference in water and DMSO crosses is entirely due to treatment or whether the effect of DMSO is apparently increased by the water control having a lower aneuploid frequency than normal.

4. **Tubulin extraction**

One explanation for the lack of success in inducing meiotic aneuploidy in *Sordaria* using spindle inhibitors could be that tubulin in *Sordaria* has very low binding
affinities for compounds such as MBC and benomyl although this is not supported by the effects of the compounds on spore germination and growth. Plating spores in medium containing various concentrations of MBC showed that growth was inhibited at MBC concentrations of 1 μM and above. Small germ tubes formed but grew no further. This suggested that mitosis is sensitive to MBC.

However, in order to investigate this suggestion attempts were made to extract tubulin from Sordaria as the reaction between Sordaria tubulin and vinblastine would also be of interest. Obtaining tubulin from lower eukaryotes is a difficult procedure due to the low concentrations of tubulin present in the cytoplasm, and further complicated by the problem of proteases which digest the tubulin during extraction (Roobol et al., 1980). It was decided, for a limited time, to attempt tubulin extraction from Sordaria using an assembly/disassembly procedure using a method proven to work in Physarum polycephalum (Roobol et al., 1980).

Firstly, porcine brain tubulin was extracted, according to the method of Dentler et al. (1975) and the assembly/disassembly reaction shown, by turbidometric analysis, to be sensitive to addition of colchicine. Extraction of Sordaria tubulin was attempted using the method of Roobol et al. (1980), with the difference that p-aminobenzamidine was used as a protease inhibitor in place of leupeptin, which was unavailable. The former inhibitor had been shown to be effective by Roobol et al. (1980). Attempts to isolate Sordaria tubulin were unsuccessful with no pellet
being formed after the third microtubule assembly cycle. Either the tubulin concentration was too low in *Sordaria* or the tubulin was not protected from digestion by p-aminobenzamidine. In an attempt to see if tubulin was being lost during the course of the extraction procedure samples were taken after each cycle of assembly and disassembly and SDS electrophoresis carried out (Laemmli, 1970). No tubulin bands were detected. It was obvious that the method of Roobol et al. (1980) was not suitable for *Sordaria* tubulin extraction. It was not practical to further develop extraction techniques during the course of this project.

5. **The effect of DMSO on aneuploidy in *Sordaria***

DMSO had been chosen as a solvent because it was widely reported as an inert solvent and had been used by others in MBC and benomyl tests and had no effect on aneuploidy (Mollet, 1976; Bignami et al., 1977). The unexpected finding that MBC and benomyl gave no increase in aneuploid frequency over the solvent control and that the aneuploid frequencies were always increased in the solvent control prompted further investigation into the action of DMSO. The results of treatment with 0.1% to 15% DMSO are shown in Table 5.6. With 15% DMSO cross fertility was reduced.

In Experiment 5* the water control gave a very surprising result with the total aneuploid frequency being $15.5 \times 10^{-4}$, which was outside the range of spontaneous frequencies normally found. However, despite being much
TABLE 5.6. Aneuploid frequencies following treatment with DMSO

<table>
<thead>
<tr>
<th>DMSO concentration %</th>
<th>Expt. 4</th>
<th>Expt. 5&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Expt. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freq. x10^-4</td>
<td>Total Asci Scored</td>
<td>Freq. x10^-4</td>
</tr>
<tr>
<td>0</td>
<td>7.4</td>
<td>67970</td>
<td>15.5</td>
</tr>
<tr>
<td>0.1</td>
<td>11.5</td>
<td>43450</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>8.8</td>
<td>56820</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>10.9</td>
<td>45670</td>
<td>8.1</td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td></td>
<td>9.2</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td></td>
<td>14.8</td>
</tr>
<tr>
<td>15.0</td>
<td></td>
<td></td>
<td>23.7</td>
</tr>
<tr>
<td>pFP A</td>
<td>34.1</td>
<td>14680</td>
<td>39.6</td>
</tr>
</tbody>
</table>

<sup>†</sup>Contamination resulted in loss of material. Only 26 aneuploid-containing asci counted.
greater than any level observed before, the variation observed in control frequencies is still much less than the thirty-fold differences described by Griffiths (1979) in a *Neurospora* system. Also in Experiment 5* there was the unusual observation that low concentration DMSO treated crosses in this experiment had much lower aneuploid frequencies than the control. There appeared to be heterogeneity in the base levels of aneuploidy, within the experiment.

Considering Experiments 4 and 5, at low concentrations of DMSO there was some increase in aneuploid frequency, but no dose-related response. At high concentrations of DMSO aneuploid frequencies were very obviously increased. At such high concentrations, the increase in aneuploidy due to DMSO is likely to be an unspecific effect due to general interference in cellular processes, rather than a specific effect on meiosis.

The variation in aneuploidy frequencies at low levels of DMSO with some statistically significant increases does not imply induction, for this a dose-related response would be expected. In Experiment 4 a linear regression was fitted to the data, but had a very high standard error and was not statistically significant (b = 1.629 ± 2.215, t2 = 0.735, P > 0.5). This variation in aneuploid levels among crosses within an experiment seems to be a consequence of DMSO treatment as such variation was not noted among repeat crosses in any one experiment for spontaneous levels of aneuploidy.
DMSO and the distribution of ascus types

Table 5.7 shows the distribution of ascus types following treatment with 15% DMSO. A 2 x n heterogeneity \( \chi^2 \) test showed no differences between the two treated crosses and the results were pooled. These pooled results were then compared to the totals for spontaneous aneuploidy and were found to be heterogeneous \( (\chi^2 = 15.4, P < 0.01) \). When the numbers are expressed as proportions of classified aneuploid-containing asci it is clear that the differences are due to increased proportions of type 1 and type 3 asci with a decrease in type 4 and little change in type 2. From Table 5.8, where the dose response data for each ascus type is shown, it can be seen that the only consistent increases in frequency are with 15% DMSO treatment. It appears that the effect of DMSO at this concentration is to increase both first and second division non-disjunction, but not to affect premature centromere division or premeiotic errors.

DMSO and recombination

The effect of DMSO on the frequency of recombination was investigated by scoring the frequency of 2+:6- asci in crosses of the grey-6 alleles, RW25 x C31. Table 5.9 shows the results obtained in crosses treated with 10% DMSO, the 15% DMSO crosses having failed. The DMSO affected the maturity of the cross and although asci were beginning to be discharged on day 10, spore colour was not fully developed. Crosses were therefore harvested and scored on day 11 and day 12 after crossing. There was no strong evidence to
**TABLE 5.7.** The distribution of ascus types following treatment with 15% DMSO.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Number (and proportion) of ascus type</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>1</td>
</tr>
<tr>
<td>5*</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Pooled</td>
<td>69 (0.70)</td>
<td>3 (0.03)</td>
</tr>
<tr>
<td>H₂O controls</td>
<td>290 (0.63)</td>
<td>25 (0.05)</td>
</tr>
</tbody>
</table>

**TABLE 5.8** Dose response data for each ascus type following DMSO treatment.

<table>
<thead>
<tr>
<th>Expt</th>
<th>DMSO conc(%)</th>
<th>Frequency of Ascus Types x 10⁻⁴</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>5*</td>
<td>1</td>
<td>5.7 1.6 0.5 0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.6 0 0.9 0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.1 0.4 1.5 0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.9 1.2 1.2 0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>17.6 0.5 4.3 0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>5.3 0.2 0.0 2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4.3 0.5 0.5 1.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.3 0.2 0.2 0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.2 0.0 0.4 0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>13.2 0.8 3.3 0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 5.9. The effect of DMSO on the frequency of recombination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Recombination</th>
<th>Cross 1</th>
<th>Cross 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% DMSO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 11</td>
<td>86/4520 1.9%</td>
<td>212/13610 1.6%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>212/12030 1.8%</td>
<td>222/19130 1.2%</td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% DMSO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 12</td>
<td>235/9650 2.4%</td>
<td>147/10970 1.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>243/13480 1.8%</td>
<td>238/15980 1.5%</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 5.10. The effect of DMSO on aneuploid frequencies in the absence of membranes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Frequency</th>
<th>Cross 1</th>
<th>Cross 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFPA (5mg/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25/33050</td>
<td>1/2340</td>
<td>4.3 x 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>7.6 x 10⁻⁴</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25/10210</td>
<td>12/4890</td>
<td>24.5 x 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>24.5 x 10⁻⁴</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO 2%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25/27730</td>
<td>14/19520</td>
<td>7.2 x 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>9.0 x 10⁻⁴</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
suggest that DMSO was affecting recombination frequency as in some cases the frequency in DMSO treated crosses was greater than in controls, in others less than in controls. The largest difference was in cross 1 day 12 and in this cross 2+:6- asci were difficult to score due to poor development of spore colour. This may explain the decrease in frequency.

The lack of effect on recombination frequency suggests that any increases in type 1 asci are not due to non-conjunction of homologous chromosomes.

**The action of DMSO - the effect of membranes**

As DMSO is an organic solvent, one possibility was that it was dissolving the cellophane membrane on which the crosses were carried out, and that the product of this reaction was responsible for the effects on aneuploid frequency. Undiluted DMSO had no obvious effects on the membrane but a further check was made by carrying out crosses without membranes. This results in much poorer crosses with smaller and fewer perithecia. Lack of membranes increased the sensitivity of *Sordaria* to DMSO, and the maximum concentration which could be tested was 2% DMSO. The yield from the crosses was poor and it was only possible to score a maximum of twenty-five aneuploid-containing asci per treatment. The results are shown in Table 5.10. It is obvious that the effect of DMSO remained the same in the absence of membranes, with the frequency of aneuploidy being slightly inflated over that in the control cross.
6. **Discussion**

The results obtained using spindle inhibitors to test for aneuploidy induction in *Sordaria brevicollis* raise many questions. The lack of effect of colchicine was not unexpected. It confirmed the insensitivity of lower eukaryotes to this compound. However, previous work on lower eukaryotes had been in mitotic systems, therefore it was interesting to note that the same effect was found in relation to meiosis. The fact that a known aneuploid inducer in mammals produced no effect in a fungal testing system demonstrates clearly the problems of extrapolation which have to be faced when electing to use a lower eukaryotic system. This can be further illustrated by the example of vinblastine. This too gave negative results but in this case it is difficult to decide whether it is because vinblastine does not induce aneuploidy when it affects spindles or whether, like colchicine, vinblastine is ineffective against lower eukaryotic tubulin. This is because vinblastine has not been reported as having been tested for aneuploid induction in any mammalian system. Also some workers report effects of vinblastine on lower eukaryotic mitosis (Flavin and Slaughter, 1974; Cappucinelli and Ashworth, 1976; Roobol et al., 1980) but lack of effect has also been found (Heath, 1975).

This extrapolation problem will always exist when testing compounds known to affect the mammalian spindle, due to the knowledge that mammalian tubulin differs from lower eukaryotes, at least with respect to colchicine binding.
It is difficult to know how to overcome this problem. The most important point is to be aware that it exists and to properly characterise potential testing systems. A combination of different rapid testing systems could then perhaps be found, for example using Drosophila and fungi, to cover a range of compounds, minimising the extrapolation problems. It seems clear that, at least for some spindle inhibitors, fungal testing systems are inadequate.

Questions of a less general nature were raised by the results obtained using MBC and benomyl. The finding that these compounds did not cause aneuploid levels to increase above those obtained with the solvent, DMSO, was very surprising, as they were known to induce mitotic aneuploidy in Aspergillus (Bignami et al., 1977). It could be that the meiotic spindle is insensitive to these compounds or that the meiotic spindle was not exposed to the compounds. This could come about by inappropriate treatment conditions. Earlier application time did not cause any change in response but it is possible that treatment at later times after fertilization may be more effective, if MBC and benomyl are rapidly metabolized by the fungus. Another procedure which could be attempted would be to grow the fungus on MBC or benomyl-containing medium or to transfer membranes on to MBC containing medium at various times prior to fertilization. Crosses without membranes could also be carried out as these could in some way interfere with the action of MBC and benomyl.
Sordaria is sensitive to MBC in the medium, spores failing to germinate or to grow properly on medium containing 1 μM MBC. It seems clear that mitosis in Sordaria is sensitive to MBC. It is possible that protoperithecia are impermeable to MBC but by early treatment of crosses MBC should have been present in the mycelium before protoperithecial formation began. If only the mitotic spindle in Sordaria is sensitive to MBC, then cross failure could still result due to failure of the nuclear divisions prior to karyogamy and so cause lack of perithecial formation. If this hypothesis were true then the mitotic spindle in the mitosis immediately following meiosis would also have to be immune to MBC as there was no evidence for failure of the post-meiotic mitosis.

Another explanation could be that concentrations of MBC and benomyl which would induce meiotic aneuploidy also disrupt mitotic divisions and that application of the compounds at fertilization result in cross failure, due to the effects on mitosis. Perhaps later application would avoid induction of cross failure and increases in meiotic aneuploidy might be observed.

As meiosis in Sordaria takes place within the ascus wall it is possible that this wall is impermeable to MBC. However treatment prior to fertilization should overcome this barrier. Perhaps the possibility that the meiotic spindles were not exposed to MBC could be checked by use of 14C labelled MBC, which is available (Davidse and Flach, 1977). However, such an experiment may be technically
difficult due to the possibility of transfer of label and/or problems of removal of unincorporated MBC to reduce the background radiation.

Attempts to study the lack of effect by analysis of tubulin were unsuccessful. Further work in this area would require a long-term project to develop suitable methods of tubulin extraction for *Sordaria*.

Another question raised by the work on MBC and benomyl was the effect of DMSO on aneuploid frequencies. The slight increase in aneuploid frequency with low concentrations of DMSO was totally unexpected, as was the increased heterogeneity of aneuploid frequencies detected within an experiment, when crosses were treated with DMSO. It seems as if treated crosses often exhibit variation in aneuploid frequency which is not the result of specific induction of a meiotic error. Perhaps this results from the effect of treatment on maturation of the ascospores, resulting in artefactual variation in aneuploid frequency.

At high concentrations DMSO caused increases in nondisjunction of chromosomes at the first and second meiotic divisions. As there was no clear dose-related response for the action of DMSO it is likely that this effect of high concentrations is not a specific action on the meiotic process, but rather a general effect on cellular function. For example, Fukui (1980) demonstrated that DMSO inhibited cell multiplication and cytokinesis *in vivo* in *Dictyostelium mucoroides*. This inhibition was attributed to DMSO inducing bundles of actin microfilaments and Fukui and Katsumaru (1979)
have shown similar effects in *Amoeba proteus* and in HeLa cells. A non-specific action of DMSO at high concentrations interfering with spindle function, would fit in with the hypothesis of Östergren (1951), as shown in Figure 5.1, as DMSO is readily soluble in water, and so high concentrations would be expected to be necessary to produce effects on spindles. That there may be some effect on spindle function is suggested by the fact that the major effect of DMSO is on asci arising through non-disjunction, either at the first or second meiotic division.
1. Introduction

The effect of X-rays on aneuploidy in Sordaria was studied for two main reasons.

a) To examine the effects of a physical agent, which would eliminate doubts about entry of the treatment into the developing ascus.

The failure to detect induction of aneuploidy by spindle inhibitors may have been due to problems in exposing the meiotic spindle to the test compound. It was therefore decided to test the effect of a physical agent. X-rays were chosen as these are well known aneuploid inducers, at least in Drosophila, with some reports of effects in fungi and mammals.

The first report of aneuploid induction by X-rays was in Drosophila (Mayor, 1921) and this has subsequently been confirmed by many workers including Anderson (1931), Traut (1964) and Parker (1969) studying the X-chromosome, and by Bateman (1968) and Clark and Sobels (1973) for chromosome 2.

Results concerning X-ray induction of aneuploidy in other organisms are much less clear cut than those in Drosophila. Parry et al. (1979, b) showed aneuploid induction by X-rays at mitosis and meiosis in Saccharomyces cerevisiae, although doses of 20 k rad and above were used. These are very high in comparison to the 250 - 4000 rad doses used in Drosophila work. Griffiths (1979) reported γ-rays increased aneuploidy
at meiosis in *Neurospora*, but no dose levels were given. Normansell (1979) demonstrated increased mitotic non-disjunction in *Aspergillus nidulans* following exposure to X-rays, at doses of 5 k rad and above. Radiation induced aneuploidy has also been studied in mammals and the results obtained have varied depending on the stage of meiosis irradiated and when the meiotic products were studied. Uchida and Lee (1974) reported X-ray induction of aneuploidy in metaphase II oocytes in mice and Szemere and Chandley (1975) found increases in aneuploidy in metaphase II spermatocytes. However, when progeny of X-ray treated males were investigated Szemere and Chandley (1975) found no increase in aneuploidy. Speed and Chandley (1981) reported increased levels of chromosome anomalies in F₁ foetuses from irradiated spermatogonia, but increases in aneuploidy alone were not statistically significant.

Russell and Saylors (1962) recovered an increased number of XO offspring following spermatocyte irradiation and Griffen and Bunker (1964, 1967) reported increased primary trisomy following X-ray treatment of mice. However, Lyon and Meredith (1966) questioned the conclusion in the 1964 report of Griffen and Bunker and suggested that the extra chromosomes were translocation products (i.e. the mice were tertiary trisomics). These doubts exist because banding techniques were not available to identify the extra chromosomes unambiguously.

If a difference in response to X-rays exists between *Drosophila* and mammals it could be because it is necessary to
treat mammals with low doses of X-rays. The doses used in the experiments mentioned above ranged from 5 rad to 700 rad. Higher doses resulted in sterility, leaving no meiotic products for analysis. The precise dose which induced sterility depended on the stage at which germ cells were irradiated, as some stages are more sensitive than others to killing by X-rays.

There are conflicting reports on the effect of X-irradiation on aneuploidy in humans. Boué et al. (1975) presented evidence which suggested that the fathers of foetuses with chromosome anomalies had higher occupational exposure to radiation than the population as a whole. However, Smithers et al. (1974) found no increase in chromosomally abnormal offspring to men who had received therapeutic radiation to testes. Uchida (1979) reviewed studies in females. These also gave conflicting results with some studies showing positive association between maternal irradiation and aneuploidy, and some negative association.

b) To investigate the mechanism of action of X-rays.

The mechanism or mechanisms involved in X-ray induction of aneuploidy are still uncertain. Most work on this problem has been carried out using Drosophila and various hypotheses have been proposed. These are that X-ray induced meiotic aneuploidy results from: reduced chromosome pairing (Savontaus, 1975); reduced crossing-over in distal regions of the chromosomes (Chandley, 1968); increased crossing-over in proximal regions (Muller, 1954); damage to
the centromere (Clark and Sobels, 1973); increased second meiotic division non-disjunction (Anderson, 1931, Bateman, 1968); damage to spindle fibres (Traut, 1970); increased chromosome stickiness (Rapoport, 1938) and/or induced chromatid interchanges (Parker, 1969). These hypotheses are not mutually exclusive and one or all mechanisms could be operating. It is also possible that the mechanisms by which radiation induces aneuploidy may not be the same in all organisms.

Considerable doubt, therefore, still exists about the mechanism of induction of aneuploidy. As the *Sordaria* systems allow the origin of aneuploidy to be inferred it was hoped to obtain useful information about the mechanism of action of X-rays, for example increased second division non-disjunction should result in an increased frequency of type 3 asci. Analysis of 2+:6- asci from grey-6 crosses may also indicate if crossing-over in the proximal regions of linkage group IV is altered. Increased crossing-over in proximal regions would also increase the detection rate of type 3 asci.

If chromatid interchange occurred as described by Parker and Busby (1973) for normal X and fourth chromosomes in *Drosophila*, then a quasi multivalent would be expected. Assuming normal chiasma formation a quasitetravalent might be formed (Figure 6.1). Segregation at meiosis of such an arrangement may not be simple. If 3:1 segregation occurred then black spores could be formed which contain chromosome rearrangements. Neither alternate or adjacent segregation
a) Possible outcomes of 2:2 segregation

1. AB/CD  
   All asci  8 abortive

2. AD/BC  
   4S6 : 2C70 : 2C70 translocation or  
   4S6 : 2 abortive : 2 abortive

3. AC/BD  
   2S6 : 2 abortive : 2C70 : 2 abortive

If the portion of the ? chromosome exchanged is not essential for spore formation then spores abortive will be black and hence asci detectable as containing aneuploid spores.

b) Possible outcomes of 3:1 segregation

1. ABC  
   4 black : 4 abortive or 2 black : 6 abortive asci

2. ABD  
   4 black : 4 abortive asci

3. ACD  
   4 buff : 4 abortive asci

4. BCD  
   4 buff : 4 abortive or 2 buff : 6 abortive asci

buff may appear black due to C70/C70 gene dosage effect.
would produce black spores. Segregation of chromosomes from a quasitetravalent in a 2:2 manner would only produce aneuploid asci, detectable in the *Sordaria* system, if a rearrangement was formed which included a dispensable tip (see Figure 6.1). Such a rearrangement has occurred spontaneously in *Sordaria* (Bond, unpublished).

2. **Irradiation**

Crosses were irradiated at a constant dose rate, with varying times of exposure. The X-ray source used was a Newton Victor 140 kVp source with unfiltered X-rays. The X-ray source was calibrated using a standardised Baldwin Farmer X-ray dose meter, 0.126 minutes were equivalent to 100 rad. Crosses were treated prior to fertilization, immediately after fertilization and twenty-four hours post fertilization. Doses used ranged from 100 rad to 5000 rad. The filtering effect of the Petri dish lid was negligible, with transmission being 98.3%. These doses were chosen as analogous to those used in mammals (100 - 200 rad) and *Drosophila* (500 - 5000 rad). Although higher doses had been used (Parry *et al.*, 1979b and Normansell, 1979) it was decided to determine the sensitivity of *Sordaria* to lower doses. Also, the 140 kVp source produces much harder (more penetrating) X-rays than those from a 50 kVp source, which was used by Parry *et al.* (1979b) and Normansell (1979).

Irradiation immediately after fertilization is most probably not irradiating meiosis, which can hardly have
started, but is justified because of the suggestion that the effect of X-rays is not short-lived. For example, irradiating serum leads to increased non-disjunction in human cell cultures (Uchida, 1979). However, it is important to realise that irradiation pre-fertilization or immediately after fertilization is not irradiation of meiotic stages.

3. Results
a) The effect of X-rays on aneuploid frequency in Sordaria

Table 6.1 shows the data obtained after irradiating Sordaria crosses with a range of doses of X-rays (0-3000 rad), either pre-fertilization (XR1 a) or post-fertilization (XR1 b). The results obtained were very variable, but none of the changes in aneuploid frequency were statistically significant, e.g. comparing the control and 3000 rad treatment in XR1b, the 't' value was 1.859. P > 0.05. There was no greater response to X-rays when both parents were irradiated (XR1 b) than when one parent was irradiated (XR1 a). The distribution of ascus types did not show any consistent change following X-ray exposure.

Although no statistically significant increases in aneuploid frequency were observed, the fact that the majority of the X-ray treated crosses had a slightly increased aneuploid frequency suggested that X-rays may have some effect and that the experiments were on too small a scale to make this statistically significant. Further crosses were therefore treated with X-ray doses of 3000 - 5000 rad,
TABLE 6.1. The effect of X-rays (100 - 3000 rad) on aneuploid frequencies in *Sordaria*

<table>
<thead>
<tr>
<th>X-ray/rad</th>
<th>Total asci</th>
<th>Overall Freq. x10^-4</th>
<th>'t' values compared to control</th>
<th>No. of Ascus Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55850</td>
<td>9.0</td>
<td>-</td>
<td>33 2 4 5 Misc. 3 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(a) Pre fertilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>52860</td>
<td>9.5</td>
<td>0.271</td>
<td>30 1 5 7 4 3 3</td>
</tr>
<tr>
<td>500</td>
<td>46040</td>
<td>10.9</td>
<td>0.955</td>
<td>31 3 6 5 4 1</td>
</tr>
<tr>
<td>1000</td>
<td>52950</td>
<td>9.4</td>
<td>0.217</td>
<td>34 4 5 - - 7</td>
</tr>
<tr>
<td>2000</td>
<td>46450</td>
<td>10.8</td>
<td>0.904</td>
<td>31 2 6 4 4 3</td>
</tr>
<tr>
<td>3000</td>
<td>40660</td>
<td>13.0</td>
<td>1.895</td>
<td>31 6 2 2 6 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Post fertilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>55850</td>
<td>9.0</td>
<td>1.645</td>
<td>26 3 7 8 3 3</td>
</tr>
<tr>
<td>500</td>
<td>44760</td>
<td>11.2</td>
<td>1.087</td>
<td>35 3 2 3 4 3</td>
</tr>
<tr>
<td>1000</td>
<td>49100</td>
<td>8.5</td>
<td>0.287</td>
<td>24 7 8 3 5 3</td>
</tr>
<tr>
<td>2000</td>
<td>59100</td>
<td>10.7</td>
<td>0.863</td>
<td>24 4 8 5 4 6</td>
</tr>
<tr>
<td>3000</td>
<td>59300</td>
<td>10.7</td>
<td>0.837</td>
<td>30 1 5 7 4 3</td>
</tr>
</tbody>
</table>

P = 0.1
P > 0.2
P > 0.5
P > 0.05
post-fertilization. The results are shown in Table 6.2. There was an increase in frequency of asci with aneuploid spores in crosses exposed to 5000 rad. However, this high frequency was due to four rosettes which contained several asci with aneuploid spores (multiple aneuploid rosettes or MAR). The contents of these are shown in Table 6.3. This clustering effect was probably due to a pre-meiotic event being amplified in the nuclear divisions prior to karyogamy and meiosis. The aneuploid-containing asci in the multiple aneuploid rosettes would not then be independently induced events. Considering each MAR as one aneuploid induction event, X-rays did not increase the overall frequency of aneuploidy when irradiation of 3000 - 5000 rads occurred around the time of fertilization. There was an increase in supposed pre-meiotic events, amplified to produce multiple aneuploid rosettes.

To investigate the effects of irradiating crosses at the time of meiosis, crosses were exposed to X-rays twenty-four hours after fertilization. At this time the majority of asci should have been going through meiosis, although due to asynchrony of ascus development some meioses would not have been irradiated. The control level of aneuploidy was \(7.1 \times 10^{-4}\) and the frequency following exposure to 5000 rad was \(9.1 \times 10^{-4}\). This increase in frequency was not statistically significant \((t = 1.22, p > 0.2)\), neither were any multiple aneuploid rosettes observed. This supported the hypothesis that the multiple aneuploid rosettes
### TABLE 6.2.
The effect of X-rays on aneuploidy in *Sordaria* (3000 - 5000 rads)

<table>
<thead>
<tr>
<th>X-R dose/ rads</th>
<th>Total Asci</th>
<th>Freq. x10^-4 of Aneuploidy</th>
<th>Freq. x10^-4 of Independent events</th>
<th>Multiple Aneuploid Rosettes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>52190</td>
<td>9.6</td>
<td>9.6</td>
<td>MAR* 6</td>
</tr>
<tr>
<td>3000</td>
<td>53110</td>
<td>9.4</td>
<td>9.4</td>
<td>MAR 5</td>
</tr>
<tr>
<td>4000</td>
<td>58490</td>
<td>9.6</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td>59360</td>
<td>14.6</td>
<td>9.1</td>
<td>MAR 1-4</td>
</tr>
</tbody>
</table>

*MAR - rosette with more than two aneuploid-containing asci.

### TABLE 6.3.
Multiple aneuploid rosettes

<table>
<thead>
<tr>
<th>Total Asci</th>
<th>Type of aneuploid-containing asci</th>
<th>Others**</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAR 1</td>
<td>120</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>MAR 2</td>
<td>80</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>MAR 3</td>
<td>20</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>MAR 4</td>
<td>170</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>MAR 5</td>
<td>10</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>MAR 6</td>
<td>120</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>

**Others - incomplete asci with at least 2+ and 2a spores.
were due to induction of a pre-meiotic event.

Despite the fact that the increase in aneuploid frequency was not statistically significant it was possible that some of the aneuploids were induced by X-rays. If these did occur, then according to the hypothesis of Parker and Busby (1973), on some occasions black spores would be expected to contain rearranged chromosomes. Asci with aneuploid spores were therefore dissected to find out if there was any evidence of rearranged chromosomes in the black spores. Black spores germinated in 33 asci. These were crossed to S6 and C70 and scored for the decreased fertility characteristic of duplications, increased spore abortion and multiple aneuploid rosettes, all of which would indicate that the spores carried a chromosome rearrangement. In no case was there evidence of any of these phenomena. Asci examined included 27 four black : four abortive asci, 2 four black : four buff asci and 4 asci with two black spores.

It therefore appeared that X-rays, at least at doses up to 5000 rad had no effect on meiotic aneuploidy in Sordaria.

b) The effect of X-rays on recombination at the grey-6 locus

Crosses of C31 x RW25 were exposed to X-rays at doses of 3000 - 5000 rad. The frequency of 2+:6- asci was counted in both control and 5000 rad crosses. The results obtained were that two hundred 2+:6- asci were observed among 8090 asci from control crosses and among 8270 asci in 5000 rad
crosses. The frequencies were 0.0247 and 0.0242 respectively. X-rays did not affect the frequency of 2+:6- ascis.

c) **Analysis of the multiple aneuploid rosettes (MAR)**

Details of the multiple aneuploid rosettes are given in Table 6.3. As it was thought that such rosettes may have arisen through pre-meiotic events, which were amplified in the mitotic proliferation phase prior to meiosis further analysis was carried out. If these results were induced by X-ray treatment none should be present in controls. Of the six found MAR1–MAR5 were in 5000 rad treated crosses, while MAR6 was found in a control cross.

**Multiple aneuploid rosette 6**

The four aneuploid-containing asci and four asci with eight buff spores were dissected. After germination disomic spores are unstable and normally the aneuploid nuclei lose one or other of the chromosomes involved in the disomy and regain the haploid state. Whenever this chromosome loss is random the resulting culture is a balanced heterokaryon containing both S6 and C70 nuclei. This will be referred to as the aneuploid breaking down when crossed. The aneuploid spores from MAR6 behaved as S6:C70 heterokaryons when crossed to S6 and C70. All C70 spores which germinated (at least one from each ascus) gave normal crosses to S6 and C70. In only one ascus did both S6 and C70 spores germinate and these also behaved as normal in crosses to S6 and C70. There was no segregation of any factor causing increased aneuploidy, that is no multiple
aneuploid rosettes were observed. It is very unusual to find more than one aneuploid-containing ascus in a rosette but the analysis gave no evidence that MAR6 contained any factor bringing about increased aneuploidy. It therefore appeared that the aneuploid asci in MAR6 were due to primary aneuploidy and had occurred in one rosette by chance.

The remaining five multiple aneuploid clumps found in X-ray treated crosses were then analysed. Three of these had identifiable chromosome rearrangements while for the two others the results were less clear. Only a preliminary analysis was carried out to demonstrate that rearrangements had occurred. The actual break points were unmapped. Details of the analysis are presented below.

**Multiple aneuploid rosette 5**

This is a very small rosette with only ten asci, which unfortunately disintegrated when transferred to another slide for dissection. Seven black spores and twelve buff spores were salvaged, but only four black spores germinated. All had the same mating type. The cultures were crossed to wild type, S6 and C70. The crosses to wild type appeared fertile but the perithecia were barren. Crosses to S6 and C70 were very poor, but the few perithecia which did form yielded some small rosettes. In all cases the asci in these rosettes segregated 4 black : 4 spore colour mutant and there was no sign of instability of black spores in 380 rosettes examined. This result is consistent
with the idea that the black spores from the original MAR5 were duplications, the black phenotype arising because the duplicated region included the buff gene. The spores would therefore contain both a C70 and an S6 allele. The phenotype of barren perithecia or perithecia with few asci is typical of cultures containing a duplication. The barren phenotype has been described for duplications in Neurospora (Perkins and Barry, 1977) and in Sordaria (Bond, 1979).

A duplication could be generated by a quasiterminal translocation or inversion or by an insertional translocation. A quasiterminal inversion could be excluded as this would not result in asci which contain 4 black : 4 abortive spores. The other two alternatives, a quasiterminal translocation or insertional translocation cannot be distinguished without appropriate markers in the translocation. The simplest assumption was that a quasiterminal translocation had occurred as this only requires two breaks and an insertional translocation requires three (Perkins and Barry, 1977).

Figure 6.2 illustrates the differing expectations when black spores from MAR5 are crossed to S6, assuming that S6 or C70 are translocated. If the translocated allele is S6 equal numbers of asci with eight buff spores and four black and four buff spores are expected, while translocation of the C70 allele would result in the majority of asci containing four black and four buff spores. The results of crosses to S6 are shown in Table 6.4(a). It can be seen that the majority of asci contained four black and four buff spores, suggesting that the C70 allele was
FIGURE 6.2 Expectations for crosses of black spores from MAR5 when S6 or C70 translocated

(i) Crosses to S6 if C70 translocated

\[
\begin{array}{c}
| \text{C70} | \text{l.g.?} | \text{S6} | \\
|-------|-------|-------|
\end{array}
\]

Resulting asci 4 black : 4 S6

(ii) Crosses to S6 if S6 translocated

\[
\begin{array}{c}
| \text{S6} | \text{l.g.?} | \text{C70} | \\
|-------|-------|-------|
\end{array}
\]

Resulting asci 4 black : 4 S6 or 4 S6 : 4 C70
TABLE 6.4. Results of crosses of black spores from MAR 5

(a) Crosses to S6

<table>
<thead>
<tr>
<th>Ascus</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4+:4b</td>
<td>17</td>
<td>18.1</td>
</tr>
<tr>
<td>2+:4b:2+</td>
<td>22</td>
<td>23.4</td>
</tr>
<tr>
<td>2b:4+:2b</td>
<td>46</td>
<td>49.0</td>
</tr>
<tr>
<td>2+:6b</td>
<td>2</td>
<td>2.1</td>
</tr>
<tr>
<td>4b:4a</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>4+:4a</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>8-</td>
<td>5</td>
<td>5.3</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>94</strong></td>
<td></td>
</tr>
</tbody>
</table>

(b) Crosses to ylo

<table>
<thead>
<tr>
<th>Ascus</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4+:4b</td>
<td>29</td>
<td>27.6</td>
</tr>
<tr>
<td>4+:4y</td>
<td>32</td>
<td>30.5</td>
</tr>
<tr>
<td>6+:2y</td>
<td>10</td>
<td>9.5</td>
</tr>
<tr>
<td>6+:2w</td>
<td>3</td>
<td>2.9</td>
</tr>
<tr>
<td>4+:2y:2w</td>
<td>8</td>
<td>7.6</td>
</tr>
<tr>
<td>4+:2b:2w</td>
<td>4</td>
<td>3.8</td>
</tr>
<tr>
<td>4+:2b:2y</td>
<td>14</td>
<td>13.3</td>
</tr>
<tr>
<td>2+:2y:4b</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>3w:2+:1b:2y</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>2y:2b:2+:2b</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>2y:2+:2w:2b</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>105</strong></td>
<td></td>
</tr>
</tbody>
</table>

b = buff, y = yellow, w = white, + = wild type (black)
= spore colour mutant
translocated. The normal frequency of 2:4:2 asci in a buff x wild type cross is 4%. In these crosses the frequency was 23.4% indicating that the buff locus had been moved to a new location.

Assuming that MAR5 arose through a translocation which generated a duplication then it should have been possible to detect linkage of C70 to a linkage group other than linkage group II. With crosses of black spores from MAR5 to spore colour mutant markers on other linkage groups, linkage of C70 to the marker would be indicated by the absence of 4 marker : 4S6 spores. The underlying reason for this is illustrated in Figure 6.3. Unfortunately the poor fertility of the cultures resulted in the failure of most of the crosses set up to determine linkage. Results of crosses to grey-6 mutants on linkage group IV showed that C70 was not translocated to that linkage group, 20% of asci being 4 grey : 4S6.

By crossing to ylo- strains it was hoped to determine whether or not the ylo locus on linkage group II was translocated along with C70. The two alternative arrangements and expectations are illustrated in Figure 6.4. It can be seen that asci containing 4 buff and 4 yellow spores are only expected if the ylo locus is not translocated and asci with 4 buff and 4 black spores are only expected if the yellow locus is translocated. The results of crosses to ylo- are given in Table 6.4(b). These results are consistent with the hypothesis that the ylo locus was translocated. The small percentage of unexpected asci with only
FIGURE 6.3 Crosses of black spores from MAR5 to spore colour mutants.

(i) Crosses to a spore colour mutant (m) on the second linkage group involved in the translocation.

<table>
<thead>
<tr>
<th>C70</th>
<th>S6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I m II</td>
<td></td>
</tr>
</tbody>
</table>

Resulting asci when no cross-overs:
- 4 black : 4 m
- 4 black : 4 S6, m

Cross-over in region I:
- 2 black : 2 m, S6 : 2 black : 2 m
- 2 black : 2 m : 2 black : 2 m, S6

Cross-over in region II:
- 2 black : 2 S6 : 4 m
- 4 black : 2 m : 2 S6, m

(ii) Crosses to unlinked spore colour mutants

<table>
<thead>
<tr>
<th>C70</th>
<th>S6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>m</td>
</tr>
</tbody>
</table>

Resulting asci:
- 4 black : 4 m
- 4 black : 4 m, S6
- 4 S6 : 4 m
FIGURE 6.4 Expectation when black spores from MAR 5 are crossed to yellow.

(i) Both buff and yellow translocated

(ii) Only buff translocated

The either/or alternatives are presumably equally likely if chromosome II assorts independently of the translocated chromosome. This may not be true if there is frequent pairing between S6 + or + ylo and the C70 + segment.

(Perhaps the only ascus to successfully complete meiosis are those in which this does not happen).

\[ y = \text{yellow}, \ w = \text{white (double mutant)} \ \ S6 = \text{buff with S6 phenotype.} \]
2 black spores may be due to immaturity of spore colour.

From the results obtained it seemed probable that MAR5 had arisen following a translocation of a portion of linkage group II, containing both the buff and yellow loci, to another linkage group. This translocation may have been a quasiterminal translocation or insertional translocation which generated a duplication. The other linkage group was unidentified but was not linkage group IV.

**Multiple aneuploid rosette 2**

All the aneuploid-containing asci and ten asci with eight buff spores were dissected from this rosette. Germiated spores were crossed to S6, C70 and wild type for further analysis.

**Black spores.** Spores from 8 asci germinated and all were unstable when crossed. However, they were not primary aneuploid spores as only the S6 component was recovered, that is, in crosses to S6 there was no segregation for spore colour.

**S6 spores.** Three germinated and all behaved as normal S6 isolates in all crosses.

**C70 spores.** When crossed to S6 and C70 a high frequency of aneuploidy was found. All rosettes contained asci with aneuploid spores. In C70 x C70 crosses the disomic spores were less dark in colour than those in S6 x C70 crosses. The rosettes contained asci with eight buff spores, eight abortive spores and type 1, 2 and 3 asci. This result was
obtained using C70 spores from 10 different asci, including one from a type 2 ascus.

Table 6.5(a) shows the results obtained from crosses of the C70 spores to S6. It was difficult to distinguish immature asci and asci with eight abortive spores and so numbers of these were not recorded. Asci from crosses of isolates 3 and 4 to wild type were therefore scored after they had discharged on to Petri dish lids. This data is given in Table 6.5(b) and it is clear that asci with eight abortive spores were formed at quite a high frequency. The results in Table 6.5(a) and (b) are compatible with there being a reciprocal translocation involving linkage group II. The fact that eight buff-spored asci were formed much more frequently than 4 buff : 4 abortive asci indicated that the break points were near the centromere (Perkins and Barry, 1977). The proposed rearrangement is illustrated in Figure 6.5. Alternate segregation of centromeres and no crossing-over between centromere and break-point would generate 8 buff-spored asci whilst adjacent-1 segregation would generate 8 abortive-spored asci. The excess of 8 buff-spored asci over those with 8 abortive spores can be explained by an excess of alternate segregation over adjacent-1 segregation. Asci with 4-:4abc could arise by crossing-over between the centromere and the break point.

The hypothesis that MAR2 arose due to a chromosome rearrangement is supported by the finding that isolate 1 crossed to isolate 3 gave apparently normal C70 x C70 crosses.
TABLE 6.5. Crosses of C70 spores from MAR 2

(a) Crosses to S6

<table>
<thead>
<tr>
<th>Isolate</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>8b</td>
<td>26</td>
<td>90</td>
<td>51</td>
<td>153</td>
<td>320</td>
<td>70.2</td>
</tr>
<tr>
<td>Type 1</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>25</td>
<td>46</td>
<td>10.1</td>
</tr>
<tr>
<td>Type 2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>14</td>
<td>3.1</td>
</tr>
<tr>
<td>Type 3</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>8</td>
<td>11</td>
<td>2.4</td>
</tr>
<tr>
<td>4b:4a</td>
<td>-</td>
<td>10</td>
<td>12</td>
<td>22</td>
<td>47</td>
<td>10.3</td>
</tr>
<tr>
<td>4+:2b:2a</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>2+:2b:4a</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>3</td>
<td>0.6</td>
</tr>
<tr>
<td>6b:2a</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>11</td>
<td>14</td>
<td>3.1</td>
</tr>
<tr>
<td>TOTAL</td>
<td>41</td>
<td>110</td>
<td>74</td>
<td>231</td>
<td>456</td>
<td></td>
</tr>
</tbody>
</table>

8 abortive asci not recorded.

(b) Crosses to wild type

<table>
<thead>
<tr>
<th>Isolate</th>
<th>3</th>
<th>4</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4+:4b</td>
<td>61</td>
<td>91</td>
<td>152</td>
<td>55.7</td>
</tr>
<tr>
<td>4+:4a</td>
<td>18</td>
<td>23</td>
<td>41</td>
<td>15.0</td>
</tr>
<tr>
<td>8a</td>
<td>20</td>
<td>60</td>
<td>80</td>
<td>29.3</td>
</tr>
<tr>
<td>TOTAL</td>
<td>99</td>
<td>174</td>
<td>273</td>
<td></td>
</tr>
</tbody>
</table>

(c) Crosses to hyaline (linkage group VI)

<table>
<thead>
<tr>
<th>Ascus</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4b:4hy</td>
<td>82</td>
<td>71.3</td>
</tr>
<tr>
<td>4+:4a</td>
<td>14</td>
<td>12.2</td>
</tr>
<tr>
<td>2b:2+:2a:2hy</td>
<td>8</td>
<td>7.0</td>
</tr>
<tr>
<td>2b:2+:4hy</td>
<td>5</td>
<td>4.3</td>
</tr>
<tr>
<td>6-:2a</td>
<td>3</td>
<td>2.6</td>
</tr>
<tr>
<td>2+:4a:2hy</td>
<td>2</td>
<td>1.7</td>
</tr>
<tr>
<td>4+:2hy:2a</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>TOTAL</td>
<td>115</td>
<td></td>
</tr>
</tbody>
</table>

+ = wild type, b = buff, hy = hyaline, a = abortive.
FIGURE 6.5 Meiotic pairing in crosses to S6 of possible rearrangements giving rise to MAR 2

a) Buff locus translocated

b) Buff locus not translocated
Crosses of isolate 3 to spore colour mutants on all other linkage groups were carried out to search for altered linkage relationships for C70. Linkage was found between C70 and hyaline on linkage group VI. The data are shown in Table 6.5(c). Spore colour mutants on each of the remaining five linkage groups segregated independently of C70 (data not shown). The linkage of C70 and hyaline suggested a reciprocal translocation involving linkage groups II and VI. The proposed rearrangement is illustrated in Figure 6.5(a) and (b). The buff locus may or may not have been translocated and the same is true of the hyaline locus.

The 4 black : 4 abortive ascis observed in crosses to S6 or hyaline can be explained by postulating 3:1 segregation of chromosomes in which one pair of homologues non-disjoin, with normal segregation of the other pair. This results in three chromosomes going to one pole and only one to the other. There are precedents for such segregation in Neurospora (Perkins and Barry, 1977) and it is suggested that 3:1 segregation occurs most frequently if the break-point is close to the centromere. This is thought to be true for MAR2. This type of segregation will also explain 4 buff : 4 abortive ascis. With reference to Figure 6.5(a) and a cross of S6 x C70 translocation, ascis with black and abortive spores would result if chromosomes ABC or ACD segregated together. If a disomic spore contains ABC then, following germination, the resulting culture will contain BC nuclei only. If the disomic spore contains ACD then the
culture will contain AD nuclei only. The recovery of the S6 component only from the black spores analysed suggested that these were the result of segregations in which ACD went to one pole. This suggested that linkage group VI non-disjoined more frequently than linkage group II. Segregation of ABD or BCD together would result in 4 buff : 4 abortive asci.

If the buff locus was not translocated (Figure 6.5(b)) then similar arguments apply except that black spores would always arise through non-disjunction of linkage group II and, to explain preferential recovery of the S6 component, the segregation must most often be ABD rather than ABC.

Premature centromere division appears increased as there is a high frequency of type 2 and type 3 asci. There is no evidence that second division segregation is affected as the numbers of type 3 asci are not in excess of type 2.

Although the positions of the break-points have not been ascertained it is clear that MAR2 is a rearrangement between linkage group II and linkage group VI, which results in the duplication of the buff locus in a significant number of meioses.

Multiple aneuploid rosette 3

This was a small rosette with two type 1 asci and one type 3 ascus. These three asci and nine eight-buff spored asci were dissected and germinated spores crossed to wild type, S6 and C70 strains.
Black spores. Only two germinated. These spores (1 and 2) were obtained from different type 1 asci and gave different results on crossing to S6 and C70. Spore 1 crosses yielded only multiple aneuploid rosettes when crossed to S6 and C70. There was an increased frequency of asci with eight abortive spores, although this was not accurately determined due to difficulty in distinguishing abortive and immature asci. The results for other ascus types are shown in Table 6.6(a).

Spore 2 gave a culture from which only the S6 component could be recovered. No multiple aneuploid rosettes were observed in crosses to S6 or C70.

Buff spores. No S6 spores and only one C70 spore (spore 3) germinated from the nine asci dissected. The culture from spore 3 was crossed to S6 and C70 and gave results similar to spore 1. These are shown in Table 6.6(a).

The results obtained with spores from MAR3 could be explained by a similar rearrangement to that in MAR2, C70 being linked to the translocation. Crosses of spore 3 to spore colour mutant markers on all other linkage groups were carried out to search for altered linkage relationships of C70. Linkage was found between C70 and grey-6 on linkage group IV. The data are shown in Table 6.6(b). Spore colour mutants on each of the five remaining linkage groups segregated independently of C70.

Proposed rearrangements are illustrated in Figure 6.6 (a) and (b). Again 3:1 segregation is proposed to explain
### TABLE 6.6. Crosses of spores from MAR 3

#### (a) Crosses to S6. Isolates 1 and 3

<table>
<thead>
<tr>
<th>Ascus</th>
<th>Spore 1 (black)</th>
<th>Spore 3 (C70)</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>8b</td>
<td>269</td>
<td>201</td>
<td>470</td>
<td>77.7</td>
</tr>
<tr>
<td>4b:4a</td>
<td>75</td>
<td>45</td>
<td>120</td>
<td>19.8</td>
</tr>
<tr>
<td>4+:4a</td>
<td>7</td>
<td>6</td>
<td>13</td>
<td>2.2</td>
</tr>
<tr>
<td>2b:2+:4a</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>353</strong></td>
<td><strong>252</strong></td>
<td><strong>605</strong></td>
<td></td>
</tr>
</tbody>
</table>

8a asci not scored.

#### (b) Crosses to grey-6. Isolate 3

<table>
<thead>
<tr>
<th>Ascus</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>4b:4g</td>
<td>179</td>
</tr>
<tr>
<td>2+:6-</td>
<td>11</td>
</tr>
<tr>
<td>4-:4a</td>
<td>51</td>
</tr>
<tr>
<td>4+:4a</td>
<td>5</td>
</tr>
<tr>
<td>2-:2a:2+:2a</td>
<td>1</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>247</strong></td>
</tr>
</tbody>
</table>

b = buff, g = grey, - = spore colour mutant (buff or grey), + = wild type, a = abortive.
FIGURE 6.6 Meiotic pairing in crosses to S6 of possible rearrangements giving rise to MAR 3

(a) Buff locus translocated

(b) Buff locus not translocated
the occurrence of asci with four black and four abortive spores. The lower frequency of these compared to MAR2 and the lack of type 2 and type 3 asci suggests that this rearrangement had a lesser effect on chromosome segregation than the rearrangement causing MAR2. The two black spores analysed gave rise to different types of cultures. There was therefore no evidence that when 3:1 segregation did occur there was any preference for the three chromosomes to include two unaltered or two rearranged chromosomes. However two samples cannot give a true indication of the likelihood of segregation patterns.

Again, as for MAR2, it is not clear whether or not the buff locus has been translocated. If the buff locus has been translocated to linkage group IV (Figure 6.6(a)) then non-disjunction of either linkage group II or IV will generate 4 black : 4 abortive asci. Since the frequency of these is comparatively low, non-disjunction of both linkage groups would necessarily be low. The high frequency of 4 buff : 4 abortive asci in the crosses would then remain unexplained. (They could arise from crossing-over giving second division segregation for the break-points but in that case the 4 buff : 4 abortive asci would be expected to have either a 2:2:2:2 or a 2:4:2 sequence of spores. Since the 2:4:2 sequence was not found this explanation appears unlikely).

If the buff locus has not been translocated (Figure 6.6(b)) then 4 black : 4 abortive asci will be generated by non-disjunction of linkage group II and 4 buff : 4 abortive asci by non-disjunction of linkage group IV. The difference
in frequency of these two ascus types could then be explained by different frequencies of non-disjunction of the two linkage groups.

Preliminary evidence therefore suggests that MAR3 has arisen through a reciprocal translocation involving linkage groups II and IV, which has a smaller effect than the MAR2 translocation, but does increase non-disjunction frequency for chromosomes involved.

Multiple aneuploid rosette 1

All the aneuploid-containing asci and six asci with eight buff spores were dissected from this rosette. Of the thirteen asci with aneuploid spores only two black spores from one ascus germinated. No buff spores germinated. The remainder of the rosette was then spread as random spores, on dissecting agar. Again no spores germinated. The only material available for analysis was, therefore, the two germinated black spores, which were crossed to wild type, S6 and C70. In crosses to wild type all asci were 4 black : 4 buff, indicating that the black spores were not recombinant and were likely to have been carrying both S6 and C70 alleles. In crosses to S6 and C70 perithecia were examined to find out if the cultures were balanced heterokaryons. In crosses to C70 the majority of perithecia contained asci segregating for spore colour. Some perithecia were difficult to classify due to immaturity. However in crosses to S6 it was clear that there was no segregation of C70 in 22/22 perithecia in crosses of one
isolate and 19/20 in crosses of the other. The remaining perithecium was clearly 1/2 segregating asci and 1/2 non-segregating asci. It appeared that there was preferential recovery of the S6 component from the cultures of the black spores. As discussed for MAR2, this could result if the black spores were tertiary disomics. With no other material available for analysis it was not possible to test this hypothesis.

**Multiple aneuploid rosette 4**

Only the black spores in the type 1 ascus germinated and three C70 spores germinated, two from eight buff-spored asci and one from a type 2 ascus.

**Black spores.** These behaved as balanced S6/C70 hetero-karyons in crosses to wild type, S6 and C70. In crosses to wild type asci were found with 4 black : 2 buff : 2 abortive spores and in crosses to S6 asci with 4 buff : 2 black : 2 abortive spores were found.

**Buff spores.** The results of crosses to S6 and wild type are given in Table 6.7(a) and (b). It can be seen that asci with 4 black : 2 buff : 2 abortive spores in crosses to wild type and asci with 4 buff : 2 black : 2 abortive spores in crosses to S6 were found at higher frequencies than in normal crosses. Considering the crosses to S6 then it is clear that type 2 and type 3 asci are found at almost equal frequencies suggesting that in these strains premature centromere division is increased. The type 1 asci are likely to be due to a double event which generated type 2 or 3 asci, as type 1 asci occur at a much lower frequency.
TABLE 6.7. Crosses of C70 spores from MAR4 to S6, wild type and ylo.

(a) Crosses to S6.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 buff</td>
<td>589</td>
<td>422</td>
<td>1021</td>
<td>999</td>
<td>3031</td>
<td>92.8</td>
</tr>
<tr>
<td>Type 1</td>
<td>1</td>
<td>-</td>
<td>7</td>
<td>1</td>
<td>9</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>18</td>
<td>31</td>
<td>18</td>
<td>86</td>
<td>2.6</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>12</td>
<td>31</td>
<td>17</td>
<td>88</td>
<td>2.7</td>
</tr>
<tr>
<td>2 or 3</td>
<td>-</td>
<td>7</td>
<td>39</td>
<td>10</td>
<td>26</td>
<td>0.8</td>
</tr>
<tr>
<td>2b:2a:4a</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td>4b:4a</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td>6b:2a</td>
<td>3</td>
<td>-</td>
<td>12</td>
<td>2</td>
<td>17</td>
<td>0.5</td>
</tr>
<tr>
<td>TOTAL</td>
<td>3267</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) Crosses to wt

<table>
<thead>
<tr>
<th>Ascus</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4+:4b</td>
<td>136</td>
<td>93.2</td>
</tr>
<tr>
<td>4+:4a</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>2+:2a:4-</td>
<td>8</td>
<td>5.5</td>
</tr>
<tr>
<td>TOTAL</td>
<td>146</td>
<td></td>
</tr>
</tbody>
</table>

(c) Crosses to ylo

<table>
<thead>
<tr>
<th>Ascus</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4b:4y</td>
<td>202</td>
<td>69.7</td>
</tr>
<tr>
<td>2+:2y:2b:2w</td>
<td>67</td>
<td>23.1</td>
</tr>
<tr>
<td>4+:4w</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>2+:2y:2a:2b</td>
<td>15</td>
<td>5.2</td>
</tr>
<tr>
<td>6-:2a</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>2-:6a</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>6+:2a</td>
<td>1</td>
<td>0.3</td>
</tr>
<tr>
<td>TOTAL</td>
<td>290</td>
<td></td>
</tr>
</tbody>
</table>

+ = wild type (black), b = buff, y = yellow, w = white, a = abortive.
Increased levels of type 2 and 3 asci is an inherited characteristic and is linked to C70 but not mating type. From a cross of isolate 1 x wild type twenty C70 spores were picked. All gave elevated frequencies of type 2 and 3 asci on crossing to S6 and both mating types were found. Crosses to spore colour mutants on each of the five remaining linkage groups gave non-parental ditypes so there was no evidence for a chromosome rearrangement involving other linkage groups.

Crosses of two of the C70 spores carrying this characteristic gave normal C70 x C70 crosses, with no evidence of increased levels of type 2 or type 3 asci. This suggested some kind of structural rearrangement may be involved.

Crosses of the spores to ylo strains showed that crossover frequency between C70 and ylo had not altered (Table 6.7(c)).

This is potentially a very interesting mutant which seems to increase levels of premature centromere division of linkage group II. As large numbers of asci with two abortive spores are not observed the effect on premature centromere division appears confined to linkage group II. No gross structural rearrangement involving another linkage group has been identified, suggesting that any rearrangement (if one is involved) of chromosome material is confined to linkage group II. The fact that C70 and ylo show unaltered linkage relationships suggest that any rearrangement does not involve a large part of linkage group II. As
apparently only centromere division of linkage group II is affected this suggests that the mutant is not altering a general controlling factor for maintaining sister chromatid association throughout meiosis I but is specific to linkage group II. As homozygous crosses for the mutant are normal a reasonable explanation would be a small chromosome rearrangement (deletion, inversion, duplication) in the centromere region of linkage group II which disrupts sister chromatid association.

Subsequent work by Stuart Imrie has shown that the mutant does map proximal to the buff locus and that recombination in the buff-centromere interval may be reduced. This supports the above hypothesis. However, more difficult to reconcile with such an explanation is his observation that the mutant is trans acting, that is both the chromosome carrying the mutant and its homologue are equally likely to undergo premature centromere division.

Maguire (1982) has suggested that a normal synaptonemal complex is necessary for the provision of normal disjunction of chromosomes right through to meiosis II. It may be that a small rearrangement in the centromere region prevents normal association of homologous chromosomes. If association in this region was necessary for maintenance of sister chromatid pairing until meiosis II, this would explain why either the homologue with or without the rearrangement could show premature centromere division.

Further investigation of MAR4 would seem worthwhile. More detailed analysis would probably require examination of crosses involving the mutant, by electron microscopy.
4. Discussion

At doses up to 5000 rad, X-rays did not influence the frequencies of meiotic aneuploidy in Sordaria. Any increases observed were not statistically significant. Obviously, this lack of effect makes it impossible to deduce the mechanism of aneuploid induction in Sordaria.

Treating crosses prior to meiosis did confirm that in Sordaria as in Drosophila (Muller, 1927), Vicia faba (Thoday and Read, 1947) and Zea mays (Anderson, 1936) to name a few examples, X-rays do induce chromosome rearrangements, shown by the multiple aneuploid rosettes. That these are due to premeiotic events is suggested by the observations that only individual perithecia are affected and that MAR's are induced in crosses irradiated prior to meiosis but not in crosses irradiated at the time of meiosis. Of the five X-ray-induced MAR, two appear to be due to reciprocal translocations, with slight evidence that the same explanation may apply to a third. Another MAR seems, from preliminary analysis, to have arisen from an insertional translocation or quasiterminal translocation leading to a duplication. The final MAR is potentially very interesting. Although it is similar to mutant strains described by other workers for example mei-322 in Drosophila (Davis, 1971) in that it promotes premature centromere division it is unique in that it acts only in the heterozygote and is trans acting.

The induction of MAR's by X-rays demonstrates that the Sordaria system could be used to search for dominant
mutations which affect meiosis. As two systems are available mutants specific to either linkage group II or IV could be found as could mutants which affected all linkage groups.

The fact that X-rays, at doses of 5000 rad administered prior to meiosis, induced chromosome rearrangements in mitotically dividing nuclei suggested that the same could happen at meiosis. However rearrangements induced in cells about to undergo meiosis would not form a cluster of asci containing the rearrangement. Individual aneuploid asci from X-ray treated crosses were examined but did not reveal any rearrangements. The effect on mitosis was quite small, with only five MAR's, their impact being due to the mitotic proliferation phase amplifying the numbers of aneuploid-containing asci. It may be that with higher X-ray doses aneuploid induction at meiosis may occur through induction of chromosome rearrangements.

This lends some support to the hypothesis of Parker and Busby (1973) that aneuploid induction arises through increased chromosome rearrangements. Their hypothesis is based on the idea of chromatid interchange leading to the formation of quasi multivalent associations at meiosis. This would not occur at mitosis, but the rearrangements detected in this project could have originated either as chromatid or chromosome rearrangements.

Parker and Busby (1973) studied the X and 4th chromosomes in Drosophila and with this system coincident aneuploidy of both chromosomes is found, with rearranged chromosomes
not necessarily being recovered in the aneuploid nuclei. In Drosophila chiasma are not formed along the fourth chromosomes and so, if a quasitrivalent of X, X and 4 form, then one fourth chromosome is left free to assort independently.

However, expectations must be slightly different in cases where most homologous pairs of chromosomes normally form chiasma. In this case a quasitetravalent will form (Figure 6.1). Now most aneuploidy would be expected to arise through abnormal segregation of chromosomes involved in this association. As discussed earlier, in the Sordaria system only 3:1 segregations (with one proviso, Figure 6.1) would produce detectable aneuploids.

From the work here it is obvious that to test these expectations with Sordaria will require treatment with X-rays at doses in excess of 5000 rad. The doses administered in this work were chosen as comparable to levels used in man, and for higher doses, in Drosophila. As Sordaria proved resistant to levels to which man and Drosophila are susceptible the problem of extrapolation between species is again raised. How valid is extrapolation when doses differing by at least an order of magnitude have to be used to produce similar levels of aneuploidy in different species? If the doses differ to such an extent perhaps the mechanism by which they produce their effect is also different. The only way to resolve this problem is to look and see what happens. The Sordaria system, if induction can be achieved,
should allow some idea of the mechanism by which the aneuploidy is arising to be obtained.

The failure to induce aneuploidy, even with the relatively high doses of X-rays used, puts Sordaria in the same category as most other organisms in which aneuploid induction is, at best, weak. Only in Drosophila do X-rays bring about large increases. Perhaps Drosophila is unique in that X-rays bring about induction by some aspect of the biology of Drosophila, peculiar to this organism. For example, perhaps the fact that the chromosomes are held together in a chromocentre facilitates chromosome rearrangements, which in turn result in aneuploidy.

The occurrence of multiple aneuploid rosettes in Sordaria indicates one of the shortcomings of random spore plating methods, in which mitotic proliferation occurs after treatment, and which have no back-up method to further analyse aneuploid induction. Multiple aneuploid rosettes would result in large numbers of spores which could grow on plating medium. This would give the false impression that meiotic aneuploidy had been increased. This kind of confusion could arise in the systems of Smith and Yorston (1981) and Griffiths (1979). A Sordaria random spore plating method would also cause such confusion, but this would be resolved by ascus analysis. Yeast random spore plating methods will not be susceptible to such confusion unless several mitotic divisions are allowed between treatment and transferring cells to meiosis-inducing medium.
CHAPTER 7: RANDOM SPORE PLATING

Using ascus analysis to screen for agents which induce meiotic aneuploidy gives information about the type of meiotic error being induced, in addition to giving the frequency of aneuploidy. However ascus analysis requires microscopic examination of all products of meiosis, which makes the time taken to score a cross quite lengthy. Also, skill is necessary to detect aneuploid products. Both the time and skill involved add to the cost of screening by ascus analysis. With a random spore plating method spores are plated out on selective medium, which only permits growth of aneuploids. A large number of meiotic products can, therefore, be screened in a short time, and little technical expertise is necessary for scoring.

Several meiotic aneuploidy testing systems based on random spore plating already exist, as outlined in Chapter 1, but most of these either do not detect second division errors of disjunction or do not distinguish first and second division errors. The exception to this is the recently published yeast system of Sora et al. (1982). With systems which do not distinguish between errors at the two meiotic divisions, it is difficult to know how any inducer is acting. Strains which could be used for either form of analysis would be very useful. The random spore plating method could be used to detect whether induction had occurred and, if positive, material from crosses of the same isolates could be examined by ascus analysis. This would reveal which classes of aneuploidy
were affected by the agent. An ascus analysis method existed in *Sordaria* and so attempts were made to develop a random spore plating method which would complement this.

Random spore plating depends on having strains in which the frequency of prototrophs generated by aneuploidy is greater than the frequency generated by recombination. This can be achieved in two ways.

a) By having chromosomes marked with a series of recessive markers, arranged so that multiple recombination is necessary to produce spores able to grow on selective medium. The arrangement of markers is shown below:

\[
\begin{array}{c}
\circ \\
\circ \\
\circ \\
\end{array} \quad a \quad b \quad + \\
\begin{array}{c}
\circ \\
+ \\
+ \\
\circ \\
\end{array} \quad c \quad + \quad d
\]

If the system is to detect both first and second division errors then the group of selective markers should not span the centromere. This is the arrangement used by Sora *et al.* (1982).

b) By having chromosomes marked with complementing auxotrophic alleles, where the frequency of recombination between alleles is less than the spontaneous aneuploid frequency. This is the basis of the method described by Smith and Yorston (1981).

In order to set up a random spore plating method there were several problems to be overcome:

1) Ensuring tight colonial growth of germinated spores.
2) Minimising the problems of clumping of spores and heterokaryosis, to ensure only colonies arising from single spores were counted as prototrophs.
3) Obtaining suitable markers and strains.

1) **Ensuring tight colonial growth of mycelium**

Colonial growth was required to allow a high plating density of spores. The rhythmic growth mutation, \( rg \), used in filtration enrichment experiments, causes the fungus to grow as a restricted colony on Vogel's (1956) N medium. This effect is particularly pronounced when galactose is the carbon source. However, this mutation proved unsuitable for random spore plating, because \( \text{escaping} \) sectors of hyphae, which were less restricted in their growth habit, resulted in colonies soon being intermingled. It was therefore decided to rely on sorbose medium, well known as a carbon source which induces restricted colonial morphology. Experiments were carried out to determine the optimum concentration of sorbose and the best sucrose:sorbose ratio to use (table 7.1). As a result of these experiments medium A (1:1 ratio and 0.5% sorbose) was chosen as giving the best combination of colony morphology and germination. This medium (0.5% SS) was used routinely in spore plating experiments.

2) **Minimising the problem of clumping and heterokaryosis**

Auxotrophic spores germinate even on minimal medium, although growth does not normally continue after formation of a short germ tube. However the possibility has to be considered that heterokaryon formation could occur if two germ tubes with complementary auxotrophic mutations met. It has been shown that heterokaryon formation does not increase with increasing plating density of spores of
TABLE 7.1  Sucrose:sorbose media trials

Plating density: 100 spores/plate  5 plates/medium
Scored 3 days after plating.

<table>
<thead>
<tr>
<th>Medium</th>
<th>% sucrose</th>
<th>% sorbose</th>
<th>ratio</th>
<th>Growing colonies</th>
<th>% germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.5</td>
<td>0.5</td>
<td>1:1</td>
<td>238</td>
<td>47.6</td>
</tr>
<tr>
<td>B</td>
<td>1.0</td>
<td>1.0</td>
<td>1:1</td>
<td>265</td>
<td>53.0</td>
</tr>
<tr>
<td>C</td>
<td>0.5</td>
<td>1.0</td>
<td>1:2</td>
<td>128</td>
<td>25.6</td>
</tr>
<tr>
<td>D</td>
<td>0.1</td>
<td>0.2</td>
<td>1:2</td>
<td>230</td>
<td>46.0</td>
</tr>
<tr>
<td>E</td>
<td>0.2</td>
<td>1.0</td>
<td>1:5</td>
<td>247</td>
<td>49.4</td>
</tr>
<tr>
<td>F</td>
<td>0.1</td>
<td>0.5</td>
<td>1:5</td>
<td>161</td>
<td>32.2</td>
</tr>
<tr>
<td>G</td>
<td>0.1</td>
<td>1.0</td>
<td>1:10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H</td>
<td>0.05</td>
<td>0.5</td>
<td>1:10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>207</td>
<td>41.4</td>
</tr>
</tbody>
</table>

Medium Appearance of colony
A Dense colonies, tight colonial growth
B Dense colonies, slightly larger than in A
C Large dense colonies, growth less compact than A or B
D Small colonies, difficult to count
E Colonies small and spindly, impossible to count by naked eye.
F As E.
G Small colonies, difficult to count
H As G.
I Control plates, glucose complete medium.
   Unrestricted growth.
Neurospora at least up to spore concentrations of $10^4$ plate (Murray, 1963). Griffiths and Delange (1977) also reported no increase in Neurospora and Priddle-Higson (unpublished) showed that spore plating density did not influence the frequency of heterokaryosis in Sordaria. The data in table 7.3c are also consistent with this observation. This suggested that the probability of two individual spores germinating and subsequently forming a heterokaryon was not great. However, the same may not be true if the units plated are not individual spores. If aggregates of spores are plated the physical proximity of the spores may facilitate hyphal fusion and heterokaryosis.

There are several factors which will influence the importance of spore aggregates in a random spore plating method: (i) the number of spores in an aggregate; (ii) the percentage germination; (iii) the probability of a clump containing spores of complementary genotype; (iv) the probability of fusion of compatible hyphae and (v) the frequency of clumps compared to the frequency of aneuploidy. These variables make it difficult to quantify the problem in any precise way. Ideally the frequency of clumps should be less than the frequency of aneuploidy and then the probability of a clump producing a growing colony could be ignored.

Steps were taken to minimise the effect of clumps by removing them. The two methods tried, whirlimixing and settlement, are illustrated in figure 7.1. Method 1

- Newmeyer (1954)
FIGURE 7.1 Method to reduce the number of clumps of spores

Method 1: Filtration and Whirlimix

Harvest spores

Filter through single muslin → Count

Filter through double muslin → Count

Whirlimix → Count

Results

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Counted after</th>
<th>No. single spores/ml</th>
<th>% units* &gt; 4 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Filter</td>
<td>Filter 1 Filter 2 Whirlimix</td>
<td>2.3 x 10^5 2.3 x 10^5 2.2 x 10^5</td>
<td>2.4 1.4 0.5</td>
</tr>
<tr>
<td>(new material)</td>
<td>Filter 2 Whirlimix</td>
<td>5.7 x 10^5 6.4 x 10^5</td>
<td>2.9 2.4</td>
</tr>
</tbody>
</table>

Method 2: Filtration and Settlement

Harvest spores

Filter through double muslin → Count

Leave to settle

5 min 10 min 15 min 30 min → Count

Results

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Counted after</th>
<th>No. single spores/ml</th>
<th>% loss single spores</th>
<th>% units* &gt; 4 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter 1 Filter 2 Whirlimix 5 min 10 min 15 min 30 min</td>
<td>4.8 x 10^5 4.2 x 10^5 3.9 x 10^5 2.9 x 10^5 2.9 x 10^5</td>
<td>- 12.5 18.8 39.6</td>
<td>3.9 1.5 0.13** 0.33</td>
<td></td>
</tr>
<tr>
<td>(old material) Whirlimix 10 min 15 min 30 min</td>
<td>Filter 2 Whirlimix 2.3 x 10^5 2.9 x 10^5 2.9 x 10^5</td>
<td>3.9 x 10^5 2.9 x 10^5</td>
<td>0.13**</td>
<td></td>
</tr>
<tr>
<td>(old material) Whirlimix 30 min</td>
<td>Filter 2 Whirlimix</td>
<td>6.4 x 10^5</td>
<td>- 100.0</td>
<td></td>
</tr>
</tbody>
</table>

* Unit = a single spore or spore aggregate. Any spores which touched were counted as an aggregate.

** Only 1 unit observed.
did not give the same result when old and new material was used. This method relies on breaking up spore aggregates and it could be that in older material the spores stick more tenaciously. Method 2 reduced the larger clumps to less than 0.4% of units plated when old material was used. This represented a reduction in the frequency of clumps and so this method was adopted for routine use, despite the disadvantage that single spores were lost during the settling time. A settling time of 10 minutes was chosen in preference to 15 minutes, as the shorter time was equally effective in removing clumps, but did not result in as great a loss of single spores. A frequency of 0.4% was still greater than the expected aneuploid frequency (ascus analysis gave estimates of 8-12 x 10^{-4} for linkage group IV). It was therefore possible that spore aggregates could pose problems for random spore plating.

Griffiths and Delange (1977) overcame this problem in two ways. They used crosses heterozygous for heterokaryon incompatibility loci and carried out visual inspection of growing colonies two days after plating, to confirm that the source was a single spore. They found that in every case where they could identify the source of the colony it arose from a single spore. In *Sordaria* visual inspection of growing colonies proved impractical. When colonies were visible with the naked eye (day 2) it was often impossible to determine their origin. Earlier examination would not have allowed identification of growing colonies. In random spore plating experiments the settling method was used to minimise the number of
spore aggregates but examination of growing colonies using a microscope was not carried out.

3) **Obtaining suitable markers**

*Sordaria brevicollis* has seven linkage groups (Chen, 1965). Ideally markers were wanted on linkage groups II or IV as aneuploidy could then be monitored for the same chromosome by ascus analysis and random spore plating. In fact these were the only two linkage groups for which more than two auxotrophic markers were available. On linkage group II, mutations marked a methionine locus, and two nicotinamide or tryptophan loci. However the markers spanned the centromere, which would have precluded detection of second division errors and the not⁰ mutations were slightly leaky, and so not suitable.

Mutations mapping to linkage group IV marked methionine uracil, pyridoxine and riboflavin loci. The latter two proved unsuitable, as neither mutant gave reliable results when tested for nutrient requirement. In addition, apparent riboflavin requiring isolates often died on subculturing, that is the spore germinated and a culture was obtained, but all attempts at subculturing failed. A greater number of mutants was therefore necessary to develop a random spore plating method and mutant hunts were carried out to obtain new auxotrophic or drug resistance markers.

**Filtration enrichment mutant hunt.** This yielded one acetate and eight methionine requiring mutants. The acetate requirement was mapped to linkage group VI and on analysis seven methionine auxotrophs turned out to be
alleles of existing loci. One methionine auxotroph (SF1) located on linkage group IV complemented an existing mutant (RD6) on that linkage group. These could represent complementing alleles or two methionine loci.

Resistance mutant hunt. Attempts were made to obtain mutants resistant to cycloheximide, acriflavine and ethionine, three antimetabolites for which resistance mutants had been obtained in *Neurospora* (Howe and Terry, 1962; Hsu, 1962; Kappy and Metzenberg, 1965). Microconidia were UV-irradiated. Spores from crosses fertilized with the microconidia were plated on medium containing the compounds at concentrations equal to, or greater than, those necessary to inhibit growth. Only one mutant was obtained, a cycloheximide resistant strain which grow on 2 μM cycloheximide. This mapped to linkage group I.

The mutants available for development of a random spore plating method were therefore the two complementing methionine requiring mutants, RD6 and SF1, and the uracil requiring mutant on linkage group IV.

4) Obtaining strains for random spore plating

For a random spore plating method to be possible strains were required in which the frequency of prototrophic recombinants was less than the spontaneous frequency of aneuploidy. It was therefore necessary to know both the recombination frequencies between RD6, SF1 and *ura* and the order of the markers. RD6 was thought to be proximal to *grey-6* (H.L.K. Whitehouse pers. comm.) and this was confirmed by dissections of asci 2:4:2 for spore colour, in crosses of *grey-6* (C31) × *met* (RD6). The
frequency of 2:4:2's was 5.1% and 7/23 had cross-overs between RD6 and C31, that is showed first division segregation for RD6 and second division segregation for C31. Thus the centromere distance of RD6 is approximately 3.5 map units.

Analysis of crosses of grey-6(C31) met(RD6) x ura and met(RD6) ura x grey-6(C31) showed that the order of loci was centromere met(RD6) ura grey-6. The data are shown in table 7.2 and this information was used to calculate approximate values for map distances the 2:4:2 frequency being 5%. The values obtained were 0.3, 0.8 and 3.9 map units for the RD6-ura, ura-C31 and RD6-centromere intervals respectively. These values are only rough estimates as the small numbers involved mean that all estimations have a large error attached. However they did show that RD6 and uracil are quite closely linked. If SF1 was as closely linked to either RD6 or ura then a random spore plating method would be feasible.

**RD6 x SF1**

The data obtained in analysis of crosses of SF1 x RD6 are shown in table 7.3. From initial work (Table 7.3a) it was clear that the two mutants were closely linked, the frequency of wild type recombinants being $3.0 \times 10^{-3}$. However this was based on a single recombinant and so further analysis was carried out by plating spores from C31 P1)6 x RW25 SF1 crosses in 0.5% SS minimal medium plus acetate (Table 7.3b). Assuming no increase in heterokaryosis with increasing plating density, then the most accurate
### TABLE 7.2

CROSSES OF C31 RD6 x \textit{ura}^{-} AND RD6 \textit{ura}^{-} x C31

<table>
<thead>
<tr>
<th>no. asci</th>
<th>8</th>
<th>2</th>
<th>1</th>
<th>Total 11</th>
</tr>
</thead>
</table>

**1. C31 RD6 x \textit{ura}^{-}**

<table>
<thead>
<tr>
<th>genotype</th>
<th>C31 RD6 +</th>
<th>C31 \textit{ura}^{-}</th>
<th>C31 RD6+</th>
</tr>
</thead>
<tbody>
<tr>
<td>ascis</td>
<td>+ + \textit{ura}</td>
<td>+ + \textit{ura}^{-}</td>
<td>+ \textit{ura}^{-}</td>
</tr>
</tbody>
</table>

**2. RD6 \textit{ura}^{-} x C31**

<table>
<thead>
<tr>
<th>genotype</th>
<th>C31 + +</th>
<th>+ RD6 \textit{ura}^{-}</th>
<th>+ + +</th>
</tr>
</thead>
<tbody>
<tr>
<td>ascis</td>
<td>+ RD6 \textit{ura}^{-}</td>
<td>C31 + +</td>
<td>C31 + +</td>
</tr>
</tbody>
</table>

Cross 1 and 2. Order to give all recombinant types by a single cross-over. RD6 \textit{ura}^{-} C31.
TABLE 7.3  Analysis of crosses of RD6 x SF1

a) Spreading spores and picking germinated spores to test.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Wild type recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD6 x SF1</td>
<td>1/100</td>
</tr>
<tr>
<td>C31 RD6 x SF1</td>
<td>0/78 grey spores</td>
</tr>
<tr>
<td></td>
<td>0/150 black spores</td>
</tr>
<tr>
<td>Total</td>
<td>1/328</td>
</tr>
<tr>
<td>Freq.</td>
<td>3.0 x 10^{-3}</td>
</tr>
</tbody>
</table>

b) Plating spores on minimal medium and counting growing colonies

Cross C31 RD6 x RW25 SF1

<table>
<thead>
<tr>
<th>Spores/plate</th>
<th>Total colonies in 5 plates</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^4</td>
<td>636</td>
<td>2.5 x 10^{-3}</td>
</tr>
<tr>
<td>10^4</td>
<td>131*</td>
<td>2.6 x 10^{-3}</td>
</tr>
<tr>
<td>5 x 10^3</td>
<td>31*</td>
<td>1.2 x 10^{-3}</td>
</tr>
<tr>
<td>10^3</td>
<td>16*</td>
<td>3.2 x 10^{-4}</td>
</tr>
<tr>
<td>10^2</td>
<td>2*</td>
<td>4.0 x 10^{-4}</td>
</tr>
</tbody>
</table>

* Some colonies picked for further analysis to determine if true or pseudo-wild type.

c) Further analysis of colonies picked in spore plating experiment crossed on to wild type.

<table>
<thead>
<tr>
<th>Genotype of daughter grey spores</th>
<th>10^2</th>
<th>10^3</th>
<th>5 x 10^3</th>
<th>10^4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1</td>
<td>14</td>
<td>13</td>
<td>6</td>
<td>34</td>
</tr>
<tr>
<td>met^-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>WT and met^-</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14</td>
<td>17</td>
<td>8</td>
<td>41</td>
</tr>
</tbody>
</table>
estimate of the frequency of prototrophs should be that based on the highest numbers, $2.5 \times 10^{-3}$. Table 7.3c indicates that plating density did not increase heterokaryon formation. The prototrophic colonies could have arisen through disomy, heterokaryosis or recombination. To determine whether they were pseudowild types or true wild types 46 colonies were picked and 41 subjected to further analysis. For the remaining five subculturing either failed, or the cultures were contaminated. As both parents of the original cross carried mutants at the grey locus, crosses of the colonies to wild types segregated for grey spore colour, and grey spores were reisolated from these crosses. If the colony was a true wild type these spores should be prototrophic and the majority (34) were in this category (Table 7.3c). Heterokaryons or disomics would have given grey spores which were methionine requiring and four such colonies were found. Three colonies yielded both wild type and methionine requiring grey spores and could have resulted from mitotic recombination in an aneuploid, or from heterokaryon formation between a recombinant and a methionine requirer.

The estimation of the frequency of wild type (WT) recombinants in this RD6 x SF1 cross depends on which of these explanations is accepted. Assuming that all the WT met colonies arose through heterokaryon formation then the proportion of wild type recombinants equals $37/41 \times 100 = 90.2\%$ and the frequency $2.3 \times 10^{-3}$. 
If mitotic recombination is assumed to be the explanation then the proportion of wild type recombinants equals $\frac{34/41 \times 100}{1} = 82.9\%$, and the frequency $2.1 \times 10^{-3}$. Whatever explanation is true the frequency of wild type recombinants is low. It was possible that RD6 and SF1 were complementing alleles at the met-2 locus on linkage group IV. Another possibility was that in Sordaria there were two very closely linked methionine loci, similar to met-7 and met-9 in Neurospora (Murray, 1970). If this were the case a met-8 locus would have to be postulated in Sordaria. To try to distinguish these possibilities the mutants RD6 and SF1 were tested on intermediates in the methionine pathway, cysteine, homocysteine and cystathionine. Neither mutant grew on any of the intermediates, therefore either explanation could still be true.

Assuming allelism, the order of the mutations could be SF1 RD6 ura or RD6 SF1 ura. Non-allelism would allow a third possibility, RD6 ura SF1. These orders are listed below with the cross that would be necessary for random spore plating.

(1) SF1 RD6 ura – RD6 x SF1 ura – (a)
(2) RD6 SF1 ura – RD6 ura – x SF1 (b)
(3) RD6 ura – SF1 RD6 SF1 x ura – (c)

As an RD6/SF1 mutant was expected to be awkward to identify attempts were made to analyse crosses (a) and (b). If order (1) was correct then cross (a) would give reduced wild type recombinants compared to cross (b) or RD6 x SF1.
FIGURE 7.2  Attempts to obtain an SF1 ura⁻ double mutant

Cross (i)  RW25 ura⁻  x  RW25 SF1

+  spread and pick spores

0 recombinants/241

Cross (ii)  RW25 ura⁻  x  RW25 SF1  Strains reisolated from cross (i)

Q

P + S

P + S + Q

Spread and pick spores  Plating random spores  Plating random spores

0 recombinants/743  0.5% SS min. medium  0.5% SS complete medium

P 36WT/7 x 10⁴  *P 8WT/308

S 32WT/7 x 10⁴  *S 32WT/438

*Q 4WT/723

* Of the WT's in cross P  3 crossed in the isolation tube.

S 25 crossed in the isolation tube.

Q All were heterokaryotic met⁻/ura⁻

No further analysis of P and S.  As such a high proportion crossed in the tube all were likely to be mixed cultures.
If order (2) was correct then cross (b) would give reduced wild type recombinants when compared to cross (a) or RD6 x SF1. If neither cross showed reduced wild type recombinants then order (3) was likely to be correct. SF1 x \textit{ura}^-  

To carry out this analysis attempts were made to isolate an SF1 \textit{ura}^- double mutant, as illustrated in figure 7.2. No recombinants were found among 241 spores. Plating on minimal medium showed that wild type recombinants were rare and plating on complete medium and analysing colonies did not yield a double mutant. A total of 2453 spores or colonies/analysed and no double mutant was obtained. It appeared that SF1 and \textit{ura} were very closely linked, more so than RD6 and \textit{ura} suggesting that order (1) was incorrect. RD6 \textit{ura}^- x SF1  

Random spore plating was therefore carried out using spores from cross (b), RD6 \textit{ura}^- x SF1. If order (2) was correct this cross should have had a much reduced frequency of wild type recombinants compared to RD6 x SF1 crosses. The results are shown in table 7.4, the estimates of prototroph frequency being variable. Unfortunately, further analysis of prototrophs gave unsatisfactory results, probably complicated by shortcomings in the technique of sampling colonies. Samples were picked on day 5 after plating into tubes of complete medium. At this stage the mycelium had grown out of the agar and it was hoped this would reduce the possibility of inadvertently
<table>
<thead>
<tr>
<th>Spores/plate*</th>
<th>Anti clump method</th>
<th>Prototrophic colonies/5 plates</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^5$</td>
<td>1</td>
<td>166</td>
<td>$3.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>$10^4$</td>
<td>1</td>
<td>5</td>
<td>$1.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>$5 \times 10^4$</td>
<td>2</td>
<td>54</td>
<td>$2.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>$10^4$</td>
<td>2, 5 min</td>
<td>17</td>
<td>$3.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>2</td>
<td>3</td>
<td>$6.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>$5 \times 10^4$</td>
<td>2</td>
<td>102**</td>
<td>$4.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>2, 10 min</td>
<td>1</td>
<td>$2.0 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

* 5 plates were used for each treatment and plating density.

** Colonies from these plates analysed further. On day 5, 66 sampled. Of these 47 crossed in the isolation tube.
picking another spore along with the mycelium. However, of 66 colonies sampled, 47 crossed in the isolation tube, indicating that the tube contained a mixed culture, with both A and a components. Table 7.5a shows the results of analysis of the first and eighth spores of asci from ten such crosses. Ten isolates which had not crossed in the tube were crossed to a grey-6 mutant (RW25), and black spores analysed from those crosses (table 7.5b). The recovery of ura^- spores was unexpected, these may have resulted from crossing-over in the original cross, or by mitotic recombination in an aneuploid. Isolates yielding both met^- ura^- and met^- spores may have arisen through heterokaryosis or from aneuploids.

From tables 7.5a and b it can be seen that the majority of prototrophic colonies contained wild type nuclei, although the analysis was complicated by the presence of other nuclear types. The frequency of recombinants was therefore not determined and the order of the three loci was still unclear. The high proportion of wild type nuclei found in prototrophs from this cross, suggests that it would be unsuitable for random spore plating.

Development of a random spore plating method was not the sole objective of this project. Although it would have been a welcome aid to the analysis of aneuploidy in Sordaria it was not essential. It became obvious that a random spore plating method was not going to be available for the initial screening of agents to be
TABLE 7.5 Analysis of colonies from RD6 ura xSF1

a) Analysis of 10 colonies which crossed in the tube.

Genotype of first and eighth spores in asci

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>met⁻/met⁺/ura⁻</th>
<th>met⁻/met⁺/ura⁻</th>
<th>met⁻/ura⁻/WT⁺/WT⁻</th>
<th>met⁻/ura⁻/WT⁺/WT⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>met⁻</th>
<th>met⁻/ura⁻</th>
<th>ura⁻</th>
<th>WT⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>3</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>7</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
studied for their effects on aneuploidy. A concentrated effort was, therefore, put into the study of these agents using ascus analysis (Chapters 4-6) and study of the random spore plating method was left in abeyance. Unfortunately time did not permit further analysis. There are, however, several approaches to try to improve the system.

1) Colonies should be picked for analysis on day 2 after plating. This would reduce the probability of mixed cultures forming in the plate and so confusing analysis. Reduced plating density would also help.

2) Picking colonies into minimal medium would maintain selection against any auxotrophic spores inadvertently picked when sampling a colony.

3) Introduction of the C/c heterokaryon incompatibility alleles into crosses would help reduce the probability of heterokaryon formation. This locus would have to be mapped first to ensure it was not on linkage group IV.

4) Analysis would be simplified if another linkage group was marked. This would allow heterokaryons and disomics to be distinguished as heterokaryons should be heterozygous for the markers on the other linkage group. Disomics should carry only one copy of the other linkage group, assuming multiple disomy is not frequent.

Attempts were made to introduce ace into either the RD6 ura or SF1 strains before the random spore plating was carried out, but unfortunately three sets of crosses failed.

5) Recently, when reisolating strains for another purpose,
six black spores were picked from a cross of RW25 ura⁻ x SF1 and one was an SF1 ura⁻ double mutant. This contrasts with the result of no double mutants found in analysis of 2453 spores or colonies analysed from a cross of RW25 ura⁻ x RW25 SF1. To explain the generation of such a mutant from the RW25 ura⁻ x SF1 cross by a single cross-over requires that ura be proximal to SF1. It therefore appears that the order of markers is RD6 ura SF1 but further analysis of the SF1 x RW25 ura⁻ cross would be necessary to confirm this. If correct, an RD6 SF1 double mutant would be required for a successful random spore plating method. This could be identified in 2:4:2's for spore colour in a cross of RD6 C31 x ura SF1. An order of RD6 ura SF1 would mean that there were two methionine loci on linkage group IV but this would not be analogous to met-7 and met-9 in Neurospora (Murray, 1970) as there is no known locus between these two methionine loci.

5. Summary

The work described here has not resulted in the development of a random spore plating method, but the potential for such a method still exists as RD6, ura and SF1 are closely linked. From preliminary evidence it appears that RD6 and SF1 are non-allelic and that the order of markers is RD6 ura SF1. If true then a new methionine locus, met-8, has to be postulated on linkage group IV. The occurrence of an SF1 ura⁻ double mutant in a sample of six spores from the cross SF1 x RW25 ura⁻ suggests that the frequency of recombination between SF1
and \textit{ura}^- is greater than that estimated from the cross RW25 SF1 x RW25 \textit{ura}^-). Further analysis is necessary to confirm this conclusion.

Analysis of growing colonies revealed that there were mixed cultures produced which included both wild type and auxotrophic nuclei. Suggestions of how to eliminate such cultures have been outlined above. Heterokaryosis between complementary auxotrophs has not been demonstrated due to the lack of markers on other linkage groups. However it does not appear to be a major problem, because prototrophic colonies do not increase in frequency with increased plating density and a high frequency of potential heterokaryons or aneuploids was not found. It may be that heterokaryons do not form between two germ tubes and that more mature hyphae are necessary, at least for one component in the heterokaryon. This may be the reason for some of the cultures which have both wild type and auxotrophic nuclei.

The potential of the system for detecting aneuploidy is summarised in figure 7.3 where random spore plating and ascus analysis are compared. The important distance in detecting second division non-disjunction is length I for random spore plating and length II for ascus analysis. The markers for random spore plating are represented by letters as the order is still uncertain. As the markers are closely linked crossing-over between them should be negligible, as far as the proportion of aneuploids detected is concerned. It can be seen that both ascus analysis and random spore plating detect only a small
FIGURE 7.3 Detecting aneuploidy using random spore plating and ascus analysis.

\[
\begin{align*}
&\text{Random spore plating} \quad \text{Ascus analysis} \\
&
\begin{array}{ll}
\text{1st division errors} & \text{2nd division errors} \\
96\% & 96\% \text{ no exchange in interval 1} \\
& \text{no disomics viable} \\
4\% & 4\% \text{ exchange in interval 1} \\
& \frac{1}{2} \text{ disomics viable} \\
\text{Total viable disomics} & 98\% \\
& 98\% \\
\end{array}
\end{align*}
\]

The proportions of aneuploid spores, or aneuploid containing asci detected are shown, taking account of crossing over in intervals 1 and 2 respectively. Crossing over between auxotrophic markers is assumed to have a negligible effect on proportions detected in random spore plating.
proportion of second division errors. The frequency of aneuploidy detected by random spore plating will be approximately half that obtained by ascus analysis. The main reason for this is that only half the meiotic products (the disomic spores) are viable in random spore plating. The total spores plated will be eight times the number of asci scored but the viable aneuploid products only four times the number of type 1 and type 4 asci and twice the number of type 2 and type 3 asci.

Figure 7.4 outlines a scheme to attempt to improve the random spore plating method.
**FIGURE 7.4.** Recommended scheme to attempt to improve random spore plating method.

Cross: Heterozygous C/c and A/a

RD6 SF1 x ura⁻ ?

↓

Harvest spores in sterile water

↓

Filter through double muslin to remove debris, perithecial walls, etc.

↓

Transfer to 2oz. McCartney bottles
Leave to settle for 10 minutes

↓

Remove supernatant
Estimate spore concentration

↓

Add spores to 0.5% SS minimal medium + acetate

↓

Plate 20ml medium/plate

↓

Count prototrophs on day 2 and 3 after plating

↓

Any further analysis:

Sample colonies on day 2 & 3
Pick into minimal medium
CHAPTER 8: GENERAL CONCLUSIONS

From the information presented in the literature review in Chapter 1 it is concluded that it is desirable to have a lower eukaryotic testing system, with which to screen for compounds which cause increases in the frequency of meiotic aneuploidy. The present study attempted to evaluate the available systems in Sordaria brevicollis for use in that role. From previous work (Bond, 1976; Bond and McMillan, 1979a,b) it seemed that the system should be useful, the ascus analysis method having the potential to reveal the meiotic error which gave rise to aneuploidy. Development of a random spore plating method would help to increase the efficiency of screening.

The work on spontaneous frequencies described in Chapter 3 demonstrated that both the buff and grey-6 systems are capable of detecting a range of cell division errors. Some variation was observed in spontaneous frequencies of aneuploidy in different experiments. This variation, although not large, is sufficient to preclude the use of historical controls for the base frequency of aneuploidy. However, the spectrum of aneuploid types observed was relatively constant.

The 2 black : 6 grey asci found at high frequency in grey-6 × grey-6 crosses, although they increase the time required for scoring aneuploid frequencies, do allow effects of agents on recombination involving linkage group IV to be investigated. For these reasons the buff system is preferred for screening for aneuploid induction, the
grey-6 system being used subsequently to investigate effects on recombination.

Treatment of both the buff and grey-6 systems with pFPA proved that it is possible to induce aneuploidy by application of an external agent. Not only can increases in the overall frequency of aneuploidy be detected, but it is possible to show that second division meiotic errors are more susceptible to induction by pFPA than are first division errors. The effect of pFPA is not linkage group specific. Together with the work on spontaneous aneuploidy these results suggested that the Sordaria systems would be suitable for aneuploid testing.

Further work, however, revealed problems. Firstly it was not possible to carry out initial screening of compounds using a random spore plating method. Development of such a method was halted due to problems in constructing strains. The discovery that one cross involved had apparently abnormally low levels of recombination, giving false information about the linkage of methionine and uracil loci on linkage group IV, may allow these problems to be settled. There is therefore still the potential for developing a Sordaria random spore plating method.

As this was not available, work on the characterisation of the Sordaria system was carried out using only ascus analysis. Here too difficulties were encountered. These included problems with the actual Sordaria system and other problems which were probably due to the fact that a lower eukaryote was being studied. There were two main
difficulties with the *Sordaria* system.

a) Variation in aneuploid levels after treatment but no induction. Following treatment with DMSO as a solvent, or with X-rays, variation was noted in aneuploid levels, when no dose-related induction was observed. This variation was perhaps due to treatment having a variable effect on spore maturation, so causing artefactual variation in aneuploid frequency. No such variation was noted in pFPA treated crosses. This may have been because no DMSO was used. If so, perhaps use of another solvent would reduce the effect. This would not explain why X-ray treated crosses also showed such variation.

Another explanation is that treatment always induces variation in background levels of aneuploidy and this will only be noticed when no induction occurs. The variation is annoying as it increases background 'noise' in the systems. However, it has not produced statistically significant changes in aneuploid frequency and so should not prevent the systems functioning as aneuploid screening methods. The slight differences are minor compared to those observed by Griffiths (1979) using *Neurospora*. I have found no reports of variation in background levels of aneuploidy in other fungal systems.

b) The lack of effect of benzimidazole compounds.

A more serious problem with the system is that it did not detect aneuploid induction by MBC and benomyl, two benzimidazole compounds known to interact with tubulin and to cause abnormal chromosome segregation in fungi.
The explanations put forward to account for this negative result mostly assume inappropriate treatment conditions were used. Such an explanation could itself prove to detract from the *Sordaria* system.

The conditions used were those previously defined by Bond and McMillan (1979a) as most suitable for treatment with pFPA. Standard sets of treatment conditions are also used with other fungal systems (Griffiths, 1979; Morpurgo *et al.*, 1979; Parry *et al.*, 1979a; Sora *et al.*, 1982). The efficiency of the *Sordaria* system would be drastically reduced if a range of different treatment conditions had to be tried for each compound tested. However, if conditions could be found which allowed detection of induction by MBC and benomyl and also by pFPA, then new standard conditions could be defined and the problem would be solved.

Much more serious would be that no conditions could be found which would allow detection of induction of aneuploidy by MBC and benomyl. This would suggest that in some way meiosis in *Sordaria* is protected from the effects of application of at least some external agents and that the system is inadequate for use in aneuploid testing.

These were not the only problems encountered using spindle inhibitors. Negative results were also obtained using colchicine and vinblastin sulphate, both known to
cause mitotic arrest in mammals. Again treatment conditions could be at fault, but a much more likely explanation is that no lower eukaryotic testing system is going to detect any aneuploid induction effects with these compounds. As discussed in Chapter 5, lower eukaryotic tubulin is known to have a much lower binding affinity for colchicine than tubulin of higher eukaryotes.

The lack of effect of these compounds on aneuploidy in *Sordaria* emphasises the problems of extrapolation when using lower eukaryotes to screen for compounds which may have adverse effects on man. Although desirable for reasons of speed and economy lower eukaryotic systems are not ideal. Negative results have been obtained here using compounds known to be positive at lower concentrations in mammals. Work with X-rays in *Sordaria* has shown that it is much more tolerant of this radiation than are *Drosophila* or man, with doses of 5000 rad not producing increases in meiotic aneuploidy. This is also true of aneuploidy in other fungal systems (Normansell, 1979; Parry et al., 1979b). Having to apply much higher doses in lower compared to higher eukaryotes also raises questions about whether the same mechanisms are involved, and about the validity of extrapolation between species.

X-ray treatment did show that the *Sordaria* systems are well suited to search for meiotic mutants induced by X-rays, with one possible mutant being found in just one experiment with 5000 rad X-ray exposure. The existence of two systems in *Sordaria* allows the search to be applied
to mutants affecting two different linkage groups. However only dominant mutations can be detected using this method.

This work supports the premise in the Introduction that no single lower eukaryotic system can be used alone as an aneuploid screening method. The problem lies in developing a suitable battery of systems which is unlikely to produce false negatives.

The overall conclusion about the possibility of the Sordaria system being useful in such a collection is, that on the evidence here, it does not seem ideally suited for such a role. However this conclusion is based almost entirely on the results obtained using spindle inhibitors, specifically chosen for study as it was thought that they might present problems. If the lack of effect of MBC and benomyl could be attributed to inappropriate treatment conditions then the main argument against Sordaria would be removed. The problem of colchicine and vinblastin sulphate would be expected with all fungal systems. Provided that the benzimidazole problem can be overcome, then the Sordaria system is as good as any lower eukaryotic system and has several points in its favour.

a) Ascus analysis allowing identification of errors
b) A potential random spore plating method to use in conjunction with ascus analysis
c) The ability to monitor aneuploidy in two linkage groups
d) The ability to monitor effects on recombination without having to test cultures for nutrient requirements.
e) No bias in favour of spores arising from non-conjunction. Methods which select for aneuploidy by complementation of a large number of auxotrophic loci do not detect aneuploids arising after recombination between any of these loci. This therefore introduces a bias towards aneuploids arising through non-conjunction.

f) The X-ray results show that the Sordaria system allows aneuploids induced by chromosome breakage prior to meiosis to be distinguished from aneuploids due to errors arising at meiosis.
APPENDIX  Composition of Media

1. **Corn Meal Agar (CMA)**
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn meal agar (Difco)</td>
<td>17 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>2 g</td>
</tr>
<tr>
<td>Yeast extract powder (Oxoid)</td>
<td>1 g</td>
</tr>
<tr>
<td>Distilled water to 1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

2. **Dissecting Agar**
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn meal agar</td>
<td>17 g</td>
</tr>
<tr>
<td>Bacto agar (Difco)</td>
<td>13 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>2 g</td>
</tr>
<tr>
<td>Yeast extract powder (Oxoid)</td>
<td>1 g</td>
</tr>
<tr>
<td>Distilled water to 1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

3. **Vogel's Minimal Medium (Solid)**
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto agar (Difco)</td>
<td>15 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 g</td>
</tr>
<tr>
<td>Vogel's (1956) salt solution</td>
<td>20 ml</td>
</tr>
<tr>
<td>Distilled water to 1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

4. **Galactose minimal medium + acetate (liquid)**
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>20 g</td>
</tr>
<tr>
<td>Bacto agar (Difco)</td>
<td>1 g</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>7 g</td>
</tr>
<tr>
<td>Vogel's (1956) salt solution</td>
<td>20 ml</td>
</tr>
<tr>
<td>Distilled water to 1000 ml</td>
<td></td>
</tr>
</tbody>
</table>
5. **Galactose complete medium** (plating medium for mutant hunt)

- Bacto agar: 18 g
- Galactose: 24 g
- Caesin hydrolysate: 6 g
- Sodium acetate: 8.4 g
- Vogel's (1956) salt solution: 24 ml
- Vitamin solution: 12 ml
- Adenine: 24 mg
- Uracil: 24 mg

Distilled water to 1000 ml

6. **0.5% sucrose/sorbose minimal medium (0.5% ss)**

- Bacto agar: 15 g
- Sucrose: 5 g
- Sorbose: 5 g
- Vogel's (1956) salt solution: 20 ml

Distilled water to 1000 ml

**Vogel's (1956) salt solution** (50 x working strength)

- Sodium citrate: 123 g
- \( \text{KH}_2\text{PO}_4 \): 250 g
- \( \text{NH}_4\text{NO}_3 \): 100 g
- \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \): 10 g
- \( \text{CaCl}_2 \cdot 2\text{H}_2\text{O} \): 5 g
- Trace elements (see below): 5 ml
- Biotin solution (25 µg/ml): 10 ml

Distilled water to 1000 ml.
### Vogel's trace elements solution

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid</td>
<td>5 g</td>
</tr>
<tr>
<td>$\text{ZnSO}_4\cdot7\text{H}_2\text{O}$</td>
<td>5 g</td>
</tr>
<tr>
<td>$\text{Fe(NH}_4\text{)}_2\text{(SO}_4\text{)}_2\cdot6\text{H}_2\text{O}$</td>
<td>1 g</td>
</tr>
<tr>
<td>$\text{CuSO}_4\cdot5\text{H}_2\text{O}$</td>
<td>0.25 g</td>
</tr>
<tr>
<td>$\text{MnSO}_4\cdot\text{H}_2\text{O}$</td>
<td>0.05 g</td>
</tr>
<tr>
<td>$\text{H}_3\text{BO}_3$</td>
<td>0.05 g</td>
</tr>
<tr>
<td>$\text{Na}_2\text{MoO}_4\cdot2\text{H}_2\text{O}$</td>
<td>0.05 g</td>
</tr>
</tbody>
</table>

Distilled water to 100 ml

### Vitamin solution

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamin</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>2.0 g</td>
</tr>
<tr>
<td>p-aminobenzoicacid</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Inositol</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2.0 g</td>
</tr>
</tbody>
</table>

Distilled water to 100 ml
REFERENCES


BOND, D.J. (1979). The genetics of a probable insertion translocation in *Sordaria brevicollis*. *Genetics* 92: 75-82.


   genetic Nomenclature - High Resolution Banding.
   trisomies and polyploids. Human Heredity 27:
   59-72.
   recombination in vegetative nuclei of Aspergillus
   recombination by certain fungicides in Aspergillus
   nidulans. Mutation Res. 51: 189-197.
   On the genetic activity of benzimidazole and
   thiophanate fungicides on diploid Aspergillus
KAPPY, M.S. and METZENBERG, R.L. (1965). Studies on the
   basis of ethionine resistance in Neurospora.
KATO, H. and YOSHIDA, T.H. (1970). Non-disjunction of
   chromosomes in a synchronized cell population
   initiated by reversal of cocemid inhibition.
KATO, H. and YOSHIDA, T.H. (1971). Isolation of
   aneusomic clones from a Chinese hamster cell line
   following induction of non-disjunction.
   Cytogenetics 10: 392-403.


