Mitotic mapping of *Schizosaccharomyces pombe**

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SUMMARY

Genetic mapping by means of mitotic haploidization (induced by para-fluorophenylalanine) and mitotic crossing-over was carried out with the fission yeast *Schizosaccharomyces pombe*. Thirty-two different genetic markers were involved in this investigation; some meiotic linkage relationships had been previously reported (Leupold, Megnet) for 16 of these loci. Mitotic haploidization experiments resulted in the genetic identification of six chromosomes in the haploid complement.

Furthermore, in an attempt to study the mechanism of action of para-fluorophenylalanine (pFPA) on mitotic haploidization, pedigree analyses were performed by micromanipulation of diploid cells growing in the presence of pFPA. Haploid cells were detected after 40 hours of contact with the analogue and many lethal pedigree branches were observed. These observations seem to agree with Käfer's (1961) and Lhoa's (1968) suggestion that mitotic haploidization in Fungi is achieved by progressive loss of chromosomes throughout cell divisions.

1. INTRODUCTION

Genetic recombination in some eukaryotic organisms may be achieved in the absence of meiosis. Two mechanisms leading to recombinant forms during mitotic divisions have been reported: mitotic crossing-over, first described in detail in *Drosophila melanogaster* (Stern, 1936), and *mitotic haploidization* in Fungi (Pontecorvo, Tarr Gloor & Forbes, 1954). Mitotic crossing-over was also found to occur in Fungi (Pontecorvo & Roper, 1952 and 1953; review: Roper, 1966) and later was successfully used for genetic mapping of *Aspergillus nidulans* (Pontecorvo & Käfer, 1958). Together with mitotic crossing-over, mitotic haploidization (Käfer, 1958 and 1961; Forbes, 1959) was also shown to be a powerful tool for genetic analysis in *A. nidulans*.

Mitotic haploidization is characterized by the absence of exchange between

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homologous chromosomes; thus, markers located on the same chromosome always segregate together. This allows identification of whole chromosomes, whereas mapping by mitotic crossing over is limited to chromosome arms only. With the discovery (Morpurgo, 1961) that para-fluorophenylalanine (pFPA) increases considerably the rate of mitotic haploidization, chromosome mapping of several fungi by haploidization was greatly simplified (Lhoas, 1961; McCully & Forbes, 1965).

In yeasts, pFPA-induced mitotic haploidization was reported in *Saccharomyces cerevisiae* (Emeis, 1966; Strömnaes, 1968) and *Schizosaccharomyces pombe* (Gutz, 1966). Mitotic crossing-over had earlier been observed in the same species, respectively by Roman & Jacob (1958) and Leupold (1958).

Although the fission yeast *Schiz. pombe* had been proved to be a suitable organism
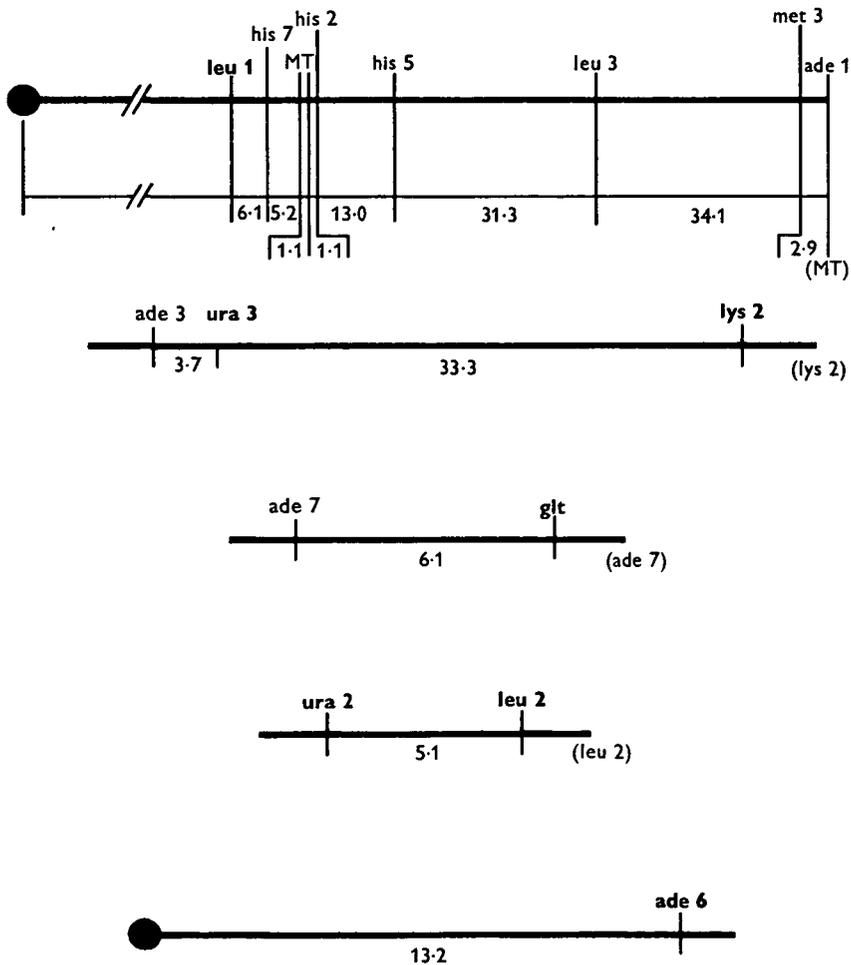
Fig. 1. Schematic representation of the various linkage relationships (in centimorgans) as determined through meiotic analysis (Leupold, Megnet: see text) in *Schizosaccharomyces pombe*. The markers shown in bold print were used in the mitotic mapping reported here. Linkage group designations used in the present study appear in parentheses on the lower right-hand side of each linkage group.

for studies on intragenic recombination and allelic complementation (e.g. Leupold, 1957; Leupold & Gutz, 1964; Angehrn, 1964; Treichler, 1964), as well as gene conversion (Gutz, personal communication), its genetic map remained fragmentary (Leupold, 1958 and personal communication; Megnet, personal communication) (Fig. 1).

The aim of the present work was to determine on how many different chromosomes the available markers were located. These studies, reported in detail elsewhere (Flores da Cunha, 1969), were conducted by means of pFPA-induced mitotic haploidization and involved 32 different genetic markers; as a result, six chromosomes in the haploid complement could be genetically identified.

Furthermore, pedigree analyses of diploid cells growing on complete medium containing pFPA provided some preliminary information on the mechanism of this analogue as an inducer of mitotic haploidization. *Schiz. pombe*, a unicellular organism, is a most suitable material for this experimental approach.

2. MATERIALS

Three mating types have been described in *Schiz. pombe*—h^+, h^- and h^{90} (Leupold, 1958). Haploid strains of mating type h^+ or h^- are heterothallic, while those carrying h^{90} are homothallic. The strains used here were obtained by Gutz,

Table 1. *Auxotrophic markers involved in the mitotic mapping experiments*

Requirement	No. of different Loci	Abbreviation
Adenine	7	*ade 1* to *ade 7*
Arginine	7*	*arg 1* to *arg 7*
Glutamic acid	1	*glt*
Histidine	3	*his 2, 5, 7*
Leucine	3	*leu 1* to *leu 3*
Lysine	5	*lys 1* to *lys 5*
Methionine	1	*met 3*
Uracil	5	*ura 1* to *ura 5*

* The markers *arg 2* and *arg 3* were found to be allelic. Only *arg 3* will be mentioned hereafter.

Leupold, Megnet, and the author, from the wild-type strains $L975\ h^+$ and $L972\ h^-$. The majority of genes were originally isolated by Leupold; those involved in the present work are shown in Table 1. Their notation is in accordance with the recommendations of the 1968 Yeast Genetics Conference at Osaka, Japan (unpublished).

Culture media: Difco Bacto-Yeast Extract (YEA: solid; YEL: liquid), Difco Bacto-Malt Extract (MEA), and minimal medium (MMA), as developed by Leupold (1955a). Sporulation medium (SPA) as reported by Angehrn (1964), and haploidization medium (YEA + pFPA) according to Gutz (1966), but with the concentration of pFPA changed to 0·1 %.

3. METHODS

(i) *Iodine vapour treatment*

Iodine vapours react with starch-like substances of ascospores and offer a simple test for their presence (Leupold, 1955*b*). Colonies with spores stain darkly (positive reaction), while those without spores become yellow (negative reaction).

(ii) *Selection and characterization of diplonts*

Although *Schiz. pombe* is a haploid organism, diploid strains can be obtained (Leupold, 1958; Gutz, 1966). These were isolated on the basis of interallelic complementation (Fincham, 1966) between certain pairs of *ade 6* heteroalleles (Leupold & Gutz, 1964). In the present work the heteroalleles $M216$ and $M210$ (or $L702$) were used to select the diplonts.* This technique automatically allowed tests for linkage with *ade 6* in all experiments. On complete medium, especially on YEA, haploid strains carrying $M216$ and $M210$ (or $L702$) form, respectively, light and dark red colonies. By interallelic complementation the diplonts $M216/M210$ (or $M216/L702$) are white and able to grow on minimal medium. Diploidy may be confirmed by the presence of azygotic asci by microscopic examination and positive iodine vapour reaction. Diplonts heterozygous for the mating type (MT) region (h^+/h^-) are unstable and undergo meiosis and sporulation at the end of their exponential growth (Leupold, 1950, 1958). Stable, non-sporulating diplonts (h^+/h^+ and h^-/h^-) arise either by mitotic non-disjunction or by mitotic crossing over between the MT region and its centromere. (A clear-cut distinction between these two mechanisms was not possible at this point, due to the lack of well-mapped markers on both chromosomes arms. The high incidence of mitotic crossing-over observed with other loci however, speaks in favour of this phenomenon.) Such stable diplonts, needed for mitotic analysis, were selected as follows.

Haploid cells of compatible mating types, obtained from 2-day cultures at 30 °C on YEA, were mixed on SPA plates. After about 15 h at 25 °C the plates were checked for zygotes by microscopic examination and the copulating mixture streaked on MMA plates and incubated for 4 days at 30 °C. From the resulting colonies, with azygotic asci, 10 vegetative cells were isolated by micromanipulation and each placed in 4 ml YEL. The liquid cultures were grown on a shaker at 30 °C for 36–72 h, after which one white culture was chosen, diluted conveniently and plated on YEA. After 4-day incubation at 30 °C all red colonies and sectors were marked and the plates were treated with iodine vapours. About 20 iodine-negative sectors and/or colonies which were white prior to iodine treatment (h^+/h^+ and h^-/h^- diplonts) were selected and streaked on YEA plates. These plates were kept at 30 °C and after 4 days they were treated with iodine vapours. From the progeny of each of the previously selected 20 sectors or colonies, one iodine-negative colony was finally chosen. These 20 colonies were placed on YEA master plates and later (after 2 days/30 °C incubation) their mating type was determined, as well as their ability to grow on MMA.

* The abbreviated notations $M216$ and $M210$ (or $L702$) will be used hereafter instead of *ade 6-M216* and *ade 6-M210* (or $L702$) respectively.

Sixty-two diplonts were utilized in the present investigation. Their collection numbers and genotypes will be mentioned only when necessary for the understanding of the experiments. Diplonts of mating type h^-/h^- were preferably used, because of negligible mutation rate to other mating types.

(iii) *Haploidization experiments*

A typical haploidization experiment, as carried out to test for linkage the majority of genes in the present work, consists of the following procedures.

A diplont, homozygous for the MT region (preferably h^-/h^-) and heterozygous for the markers in question (in trans configuration), was synthesized according to the techniques above. This diplont was, then, plated on YEA + pFPA and incubated at 30 °C. After 8 days one plate was washed off with sterile saline solution (0·85 % NaCl), and the suspension was plated on YEA to obtain 100 to 150 colonies per plate. After 4 days at 30 °C 100 colonies were isolated and tested by replica-plating (Lederberg & Lederberg, 1952) for their nutritional requirements.

Usually, 50 colonies carrying *M216* (light-red) and 50 carrying *M210* or *L702* (dark-red) were isolated. Red colonies were chosen because they provide good evidence that the process of haploidization is either initiated, the clones being monosomic for the *ade 6* chromosome, or completed, the clones being true haplonts. Visual identification, through the different shades of red, made the selection of *M216* and *M210* colonies easy in most cases. However, a haploid strain carrying, in addition to an *ade 6* allele, another gene which blocks the biosynthesis of adenylic acid at an earlier step, will form white colonies. This situation arose in all experiments involving the genes *ade 4* and *ade 5* and in these cases identification of the *ade 6* alleles was achieved by backcrosses with the parental *M216* and *M210* strains (Heslot, 1960). Backcrosses were also made whenever it became necessary to distinguish different genes with the same phenotypic expression.

Once the phenotypes of the 100 mitotic segregants was established, statistical analysis could be accomplished and the decision as to *linkage* or *no linkage* (free segregation) between the genes tested could be made.

(iv) *Criteria for mitotic linkage*

Absence of recombinant phenotypes among the haploid products of a haploidization experiment indicates mitotic linkage between the markers tested (i.e. these are located in the same chromosome pair). In principle, haploidization experiments should yield definitive results as to either linkage or no linkage. In practice, however, the occurrence of incomplete haploidization, mitotic crossing-over prior to haploidization, and mitotic nondisjunction have to be taken into account as possible sources of error.

To distinguish between linkage and free segregation, the results of the haploidization experiments were evaluated in the following way. From the obtained allele frequencies* the theoretical frequencies of each progeny class were calculated

* The allele frequencies deviate, in general, from a 50:50 ratio due to different growth rate of the segregants.

under the assumption of free segregation. The observed number of segregants in each class was then compared with the above Null Hypothesis (no linkage) by χ^2 tests (Green, 1963). When P values larger than 0·05 were obtained, the markers in question were considered to be located on different chromosomes.

(v) *Pedigree analyses*

In order to determine how soon haplonts can be detected after cells were put in contact with pFPA, pedigree analyses of diploid cultures growing on YEA + pFPA layers were carried out. These experiments were performed according to the techniques developed by Haefner (1967*b*). Uniformly thin layers ($\sim$ 0·8 mm) of solid medium were obtained by means of a simple apparatus (Haefner, 1967*a*) and placed on cover glasses for use in the micromanipulation procedures. Glass micro-needles and a pneumatic de Fonbrune micromanipulator were used. The cells were checked by microscopic examination every 2 h for 40 h. The preparations were kept at 30 °C during the periods between observations. After several cells had undergone at least four consecutive mitotic divisions, the layers were transferred to culture dishes containing YEA + 0·1 % phenylalanine, in order to counteract the effects of pFPA (Munier & Cohen, 1959). The colonies that developed after incubation of the layers on the plates at 30 °C for 4 days were further analysed.

Information about the ploidy of such clones was obtained through the following procedure: when two diplonts are crossed on YEA at 30 °C, some eight-spored asci are formed; in crosses of a diplont with a haplont not more than six spores per ascus are produced. This phenomenon was named 'twin meiosis' (Gutz, 1967*a*, *b*); it was found that the eight-spored asci result from zygotes in which plasmogamy is not followed by karyogamy, the two parental nuclei undergoing meiosis separately.

This test allows distinction between haploid and diploid strains. It is not yet known, however, how aneuplonts behave in such crosses. It should be emphasized at this point that the terms 'haploid' and 'diploid', as used throughout this paper, refer respectively to the monosomic or disomic condition of *all* chromosomes marked in a given experiment. This criterion was adopted because of lack of information about the precise number of chromosomes in *Schiz. pombe* (necessary to define haploidy in strict terms) and because of the impossibility of working with more than three auxotrophies per diplont (see below).

4. RESULTS

(i) *General design of the experiments*

The majority of markers used in the present studies were located by mitotic haploidization. All diplonts synthesized carried, in addition to the complementing *ade 6* heteroalleles, only two more heterozygous genes in repulsion. This was done because *Schiz. pombe* strains having more than three different auxotrophies in general do not grow on supplemented minimal medium, and grow very poorly even on complete medium (YEA). Therefore, all genes investigated had to be tested in pairs, and were at the same time tested against *ade 6*.

As pointed out in Methods, strains homozygous for the MT region had to be used for the haploidization experiments. Due to mitotic crossing-over between the MT region and its centromere, such segregants will be also homozygous for all markers distal to the MT region. In the process of selection of h^-/h^- or h^+/h^+ diplonts, three genes (*arg 4*, *arg 5* and *lys 4*) were found to be linked to the MT region.

The haploidization experiments were done according to the following scheme. All unlocated genes were first tested against the *lys 2* linkage group; two markers on this linkage group were used as testers, *lys 2* and *ura 3*. All genes found to be linked to either one of these were not tested against other markers. The remaining unlocated markers were then tested against *glt* (on *ade 7* linkage group). All the markers which showed no linkage with *glt* were then tested against each other.

This design reduced considerably the number of haploidization experiments, since several markers were eliminated throughout the consecutive steps.

The final results of the haploidization experiments are summarized in Fig. 3 at the end of this chapter. The different markers are already listed according to linkage groups. This arrangement was devised to facilitate their grouping into chromosomes (see Discussion).

(ii) *Linkage with the MT region*

(A) From diplont 13 (h^+/h^- *M216/M210 arg 4/+ + /lys 2*) eleven iodine-negative yellow colonies were isolated and tested for their mating type and nutritional requirements. Of those, 10 were h^-/h^- and prototrophic, the other was h^+/h^+ and auxotrophic for arginine. These data were confirmed by 12 iodine-negative colonies isolated from diplont 37 (h^+/h^- *M216/M210 + /leu 2 arg 4/ +*)—four of those were h^-/h^- and prototrophic, eight were h^+/h^+ and auxotrophic for arginine.

(B) From diplont 14 (h^+/h^- *M216/M210 arg 5/+ + /lys 2*) eight iodine-negative clones were obtained—one was h^-/h^- and prototrophic, seven were h^+/h^+ and arginine requiring.

(C) Among ten iodine-negative colonies isolated from diplont 4 (h^+/h^- *M216/ M210 lys 4/+ + /ura 3*), three were shown to be h^-/h^- and prototrophic, seven h^+/h^+ and lysine dependent. These results were confirmed by twelve iodine-negative clones obtained from diplont 27 (h^+/h^- *M210/M216 leu 2/+ + /lys 4*)—eight were h^+/h^+ and prototrophic, four were h^-/h^- and auxotrophic for lysine.

These data indicate that the genes *arg 4*, *arg 5* and *lys 4* and the MT linkage group are located on the same chromosome arm. They are located either distal to the MT region, or proximal but so near to it that the sample analysed was not large enough to allow detection of recombinants between the proximal genes and the MT region.

(iii) *Location of genes by mitotic haploidization*

(A) According to experimental results in *Aspergillus*, the lack of genetic exchange between markers on homologous chromosomes during mitotic haploidization will result in the absence of recombinant types among the resulting haploid segregants (Käfer, 1958). In order to confirm the haploidization effect of pFPA in

Schiz. pombe (Gutz, 1966) and to test the *en bloc* segregation of linked markers, a control experiment was conducted with a diplont carrying the markers *leu 2* and *ura 2*, known through meiotic analysis to be 5·1 centimorgans apart (Leupold, personal communication). The genotype of this diplont H30 was h^-/h^- *M216/M210+/leu 2 ura 2/+*. The number of segregants after pFPA-induced haloidization is shown in Table 2.

Table 2. *pFPA-induced red segregants of diplont H30*
(h^-/h^- *M216/M210+/leu 2 ura 2/+*)

	URA 2		ura 2		
	M210	M216	M210	M216	Totals
LEU 2	4	0	20	30	54
leu 2	26	20	0	0	46
Totals	30	20	20	30	100

Table 3. *pFPA-induced red segregants of diplont H6*
(h^-/h^- *M216/M210+/lys 2 ura 1/+*)

	URA 1		ura 1		
	M210	M216	M210	M216	Totals
LYS 2	0	0	38	36	74
lys 2	12	14	0	0	26
Totals	12	14	38	36	100

Four clones prototrophic for uracil and leucine were found in this experiment. Because they were thought to be recombinants for *ura 2* and *leu 2* these strains were crossed with an h^+/h^+ diplont. In all four cases, eight-spored asci were formed (twin meiosis), and therefore the strains were considered either as still being diplonts or at least disomic for the *ura 2* and *leu 2* chromosome (incomplete haploidization). If they were diplonts, their red colour can be due to homozygosity for the *ade 6* heteroalleles, caused by mitotic crossing-over between the *ade 6* locus and its centromere. This event may result in *M210/M210* or *M216/M216* adenine-requiring red strains. Because the twin meiosis test has not yet been clearly defined for aneuplonts, the possibility of finding aneuploid strains monosomic only for the *ade 6* chromosome has to be considered. Such red strains would be prototrophic for the other heterozygous markers and auxtrophic for adenine. These phenotypic prototrophs for requirements other than adenine were found in some experiments. As it was not possible to establish their ploidy in all those cases, they were counted as haplonts for statistical purposes.

It is obvious from the results shown in Table 2 that a χ^2 test could be omitted in this case, since, by mere chance, the allele frequencies were very close to the ideal 50:50 ratio.

In other experiments, however, the allele frequencies differed considerably from the 50:50 ratio. An example of such situation was found in the haploidization of

diplont H 6 (h^-/h^- $M216/M210+/lys\ 2\ ura\ 1/+$) (Table 3). A χ^2 test was used in these cases in order to test for linkage.

Forty-four haploidization experiments showed clear-cut results as above.

(B) Mitotic haploidization of diplonts H 7 (h^-/h^- $M216/M210+/lys\ 2\ ura\ 2/+$) and H 76 ($h^+/h^+$ $M216/M210\ ade\ 4/+ +/ade\ 2$), performed in order to test for mitotic linkage between *lys 2* and *ura 2* and between *ade 4* and *ade 2*, respectively, yielded the following result: segregants of 'parental' phenotype ($+ -$ and $- +$) were lost in both cases; only 'recombinant' forms ($+ +$ and $- -$) were observed. Table 4 shows these results for diplont H 7; diplont H 76 produced similar results.

Table 4. *pFPA-induced red segregants of diplont H 7*
(h^-/h^- $M216/M210+/lys\ 2\ ura\ 2/+$)

	URA 2		ura		
	M210	M216	M210	M216	Totals
LYS 2	28	26	0	0	54
lys 2	0	0	22	24	46
Totals	28	26	22	24	100

Table 5. *pFPA-induced red segregants of diplont H64*
(h^+/h^+ $M216/M210\ ura\ 5/+ +/glt$)

	GLT		glt		
	M210	M216	M210	M216	Totals
URA 5	0	0	0	0	0
ura 5	50	50	0	0	100
Totals	50	50	0	0	100

As only two classes of haploid segregants were found in both experiments, the possibility of mitotic linkage between the markers tested (*lys 2* to *ura 2*) and *ade 4* to *ade 2*) had to be considered. Most probably mitotic crossings-over had occurred prior to haploidization in these diplonts, leading to cis configuration between the markers in question. This explains the presence of only $+ +$ and $- -$ haploid segregants and therefore linkage between *lys 2* and *ura 2* and between *ade 4* and *ade 2* could be confirmed.

(C) Total loss of one allele was observed in ten experiments. Two different situations arise at this point:

(I) Only two classes of progeny are found: this was observed in two experiments. Table 5 summarizes the results obtained with diplont H 64; H 96 showed similar behaviour.

These results indicate mitotic linkage between the genes tested (other than *ade 6*). Such cases may be due to either one of the following events.

1. Due to selective disadvantage certain strains will be selected against and one allele may be lost. If the genes in question are located on the same chromosome pair, the loss of an allele will cause the concomitant loss of the other linked allele

located on the same chromosome. Thus, for instance, in the haploid segregants of a diplont $a^+ b^-/a^- b^+$, the loss of a^- will result in the elimination of all b^+.

2. Due to mitotic non-disjunction (Pontecorvo & Käfer, 1958) prior to haploidization, the diplont plated on YEA + pFPA would have been $a^+ b^-/a^+ b^-$. (On complete medium the phenotype b^- of this diplont remained undetected.) Subsequent haploidization of such a strain would produce only $a^+ b^-$ types, a^- and b^+ being lost again. Both cases 1 and 2, however, clearly indicate mitotic linkage.

Table 6. *pFPA-induced red segregants of diplont H25*
$(h^-/h^- \ M210/M216 + /lys \ 2 \ leu \ 2/ +)$

| | LEU 2 | | leu 2 | | |
	M210	M216	M210	M216	Totals
LYS 2	2	1	0	0	3
lys 2	48	49	0	0	97
Totals	50	50	0	0	100

Table 7. *pFPA-induced red segregants of diplont H36*
$(h^-/h^- \ M216/M210 + /leu \ 2 \ arg \ 3/ +)$

| | ARG 3 | | arg 3 | | |
	M210	M216	M210	M216	Totals
LEU 2	41	38	0	0	79
leu 2	9	12	0	0	21
Totals	50	50	0	0	100

The results obtained with four diplonts (diplont H25 was taken as example; see Table 6) can be included in this category (I). Although a total of 10 clones phenotypically prototrophic for the nutritional requirements other than adenine were observed altogether in all four experiments, these segregants were considered as aneuplonts, i.e. disomic for the chromosome involved. These cases, however, can only be explained by selective disadvantage caused by the loss of an allele (case 1 above).

(II) Four classes of progeny are found: haploidization of four diplonts yielded this result (diplont H36, Table 7, was chosen as an example of this category).

In these four different experiments, one allele (*arg 3*, *ura 5* or *glt*) was completely lost. The markers *arg 3* and *ura 5* could be mapped unambiguously in further experiments.

This could not be achieved, however, with respect to *glt*. It has been found that strains carrying the marker *glt* show considerable decrease in viability (Flores da Cunha, 1969); the loss of the *glt* allele due to selective disadvantage, in the present experiments, seems to be the most probable explanation. Based on the results of other experiments (see Fig. 3) and taking into account the conclusions above, *glt* was considered not linked to *lys 2* or *arg 6*, but on the same chromosome as *ura 5*.

(iv) *Linkage of arg 1 to ade 6*

Among all genes tested in this work, only one, *arg 1*, showed mitotic linkage with *ade 6* (see Fig. 3). As this was the only case of linkage with *ade 6*, and because *ade 6* is one of the best studied genetic markers of *Schiz. pombe*, a further experiment was devised to confirm this finding: a diplont of genotype h^-/h^- *M210/M216 arg1/*+ was plated on YEA and, after 4-day incubation at 30 °C, 100 red colonies (originated through mitotic crossing-over between *ade 6* and its centromere) were isolated. Among those, all (64) light-red colonies (*M216*) were arginine independent and all (36) dark-red (*M210*) required arginine. These results indicate that *arg 1* is located on the same chromosome arm as, and distally to, *ade 6*.

(v) *MT linkage group*

At this point of this investigation the MT linkage group (see Fig. 1), with three new markers added through mitotic crossing-over analysis, had not yet been located in any of the previously characterized chromosomes. The use of heterozygous markers of the MT linkage group in haploidization experiments presented some difficulties—when h^-/h^- or h^+/h^+ diplonts were obtained, through mitotic crossing-over, all markers distal to the MT region became homozygous. The most proximal gene, *leu 1*, is 11·3 map units from the MT region, and usually will also be homozygous. It was possible, however, through selection on MMA, to obtain a few diplonts homozygous for the MT region and still heterozygous for *leu 1*. Such strains, with additional appropriate markers, were used in further haploidization experiments to determine the position of the MT linkage group.

In the haploidization experiments reported above, genes *arg 3*, *arg 6* and *ade 5* were not found to be linked to any of the previously known fragments of the meiotic map, nor to each other; *leu 1*, as representative of the MT linkage group, was tested against these three markers, and against *lys 2* (from *lys 2* linkage group) and *ura 5* (from *ade 7* linkage group).

Haploidization of the appropriate diplonts showed that *leu 1* segregates independently of *arg 3*, *arg 6*, *ade 5* and *lys 2*, but segregates *en bloc* with *ura 5*.

Furthermore, no linkage was found between *leu 1* and *ade 6*, whose alleles *L702* and *M216* were included in all diplonts analysed.

In conclusion, through linkage with *ura 5*, the MT linkage group was found to be located on the same chromosome as the *ade 7* linkage group.

(vi) *Pedigree analysis*

A preliminary experiment was conducted in order to determine the approximate time of action of pFPA as a haploidizing agent. A suspension of diploid cells from a h^-/h^- *M210/M216* culture in exponential growth phase was streaked on 12 thin layers of YEA + pFPA. Four cells were isolated on each preparation and the daughter cells of each division were immediately separated. This procedure was continued until 19 of the initial 48 cells had undergone four consecutive mitoses. In between the observations the preparations were kept at 30 °C.

Among 165 viable (colony-forming) cells of the 4th generation, only four pro-
duced red colonies. These were tested by twin meiosis techniques—no eight-spore
asci were found after crossing with a diplont of compatible mating type. This
eliminates the possibility of the tested colonies being diploid.
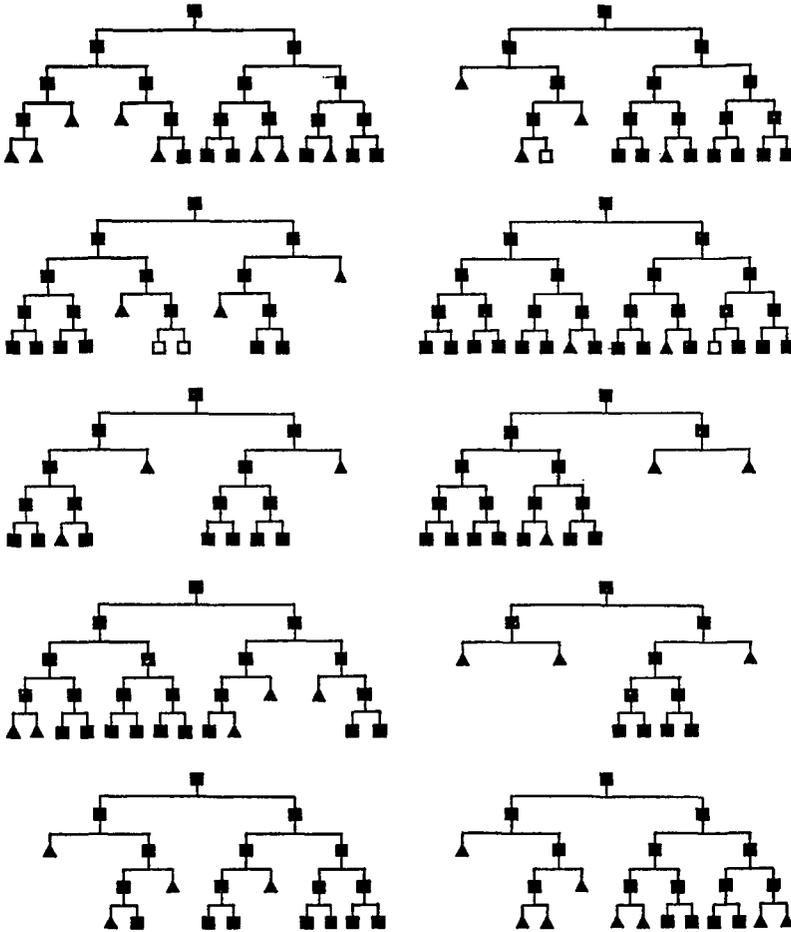
Fig. 2. ■ Viable diploid cell*; □ viable haploid cell*; ▲ non-viable cell†. (*Viable
cells = cells capable of dividing. Viability of fourth generation cells, bottom line of
each pedigree, was defined by colony-forming ability; † non-viable cells = cells
incapable of dividing).

Although these experiments were carried out only with 48 initial diploid cells, a
few conclusions can be drawn from the 19 pedigrees established (of which 10
examples are shown in Fig. 2):

The doubling time is increased, in the presence of pFPA, to 4–5 h, whereas the
normal doubling time on YEA is 2 h.

Haploid colonies were detected as early as the 4th consecutive mitosis, i.e. after
about 40 h of contact with pFPA.

Many lethal pedigree branches were observed in the presence of pFPA.

Control experiments were not performed, since data were available from Gutz & Angehrn (personal communication). These authors established pedigrees of h^{90}/h^- diploid strains on YEA. From a total of 1559 cell divisions followed by pedigree technique, 1504 divisions yielded two colony-forming cells and 55 divisions produced on colony-forming and one non-viable cell. Analysis of the 19 pedigrees obtained in the present investigation resulted in the following figures: from a total

| I | | | | | | | | | | | II | | | | III | | IV | V | VI | | Chromosomes |
ura 3	lys 2	leu 2	ura 2	ade 2	ade 4	lys 1	lys 3	lys 5	ura 1	ura 4	glt	arg 7	leu 1	ura 5	ade 6	arg 1	arg 3	arg 6	ade 5	Genes	
	●				●	●	●								○					ura 3	
		●	●	●	●				●	●	○	○	○	○	○	○	○	○	○	lys 2	
			●		●	●	●								○		○			leu 2	
															○					ura 2	
					●									○	○				○	ade 2	
											○				○					ade 4	I
															○					lys 1	
															○					lys 3	
															○					lys 5	
															○					ura 1	
															○	○				ura 4	
												●	●		○			○	○	glt	
															○		○			arg 7	II
														●	○		○	○	○	leu 1	
															○	○				ura 5	
																●	○	○	○	ade 6	III
																				arg 1	
																		○	○	arg 3	IV
																			○	arg 6	V
																				ade 5	VI

Fig. 3. Linkage relationships between all markers tested. Data obtained by pFPA-induced mitotic haploidization: ● linkage; ○ no linkage.

of 221 cell divisions, 169 produced two colony-forming cells and 52 produced non-viable cells (40 yielded one viable and one non-viable cell, 12 gave rise to two non-viable cells). Comparing, in summary, the two sets of data, diplonts growing on YEA produced 3·5 % deficient divisions, while those growing on YEA + pFPA gave 23·5 % deficient divisions.

5. DISCUSSION

(i) *Interpretation of the mitotic data*

Thirty-two different markers were involved in the present mapping studies of *Schizosaccharomyces pombe*; some meiotic linkage relationships had previously been reported (Leupold, 1958 and personal communication; Megnet personal communication) for 16 of these loci (see Fig. 1). The remaining genes were mapped by mitotic haploidization (13) and by mitotic crossing-over (3). An evaluation of the results obtained here (see Fig. 3) shows that the 32 genes involved in the present work are located in six different chromosomes (Table 8).

Table 8. *Chromsome map of* Schiz. pombe *involving 32 genetic markers**

Chromosome I

```
ade 3 ⎫
ura 3 ⎪ Leupold,
lys 2 ⎬ personal            ⎫
ura 2 ⎪ communication       ⎪
leu 2 ⎭                     ⎬
ade 2                       ⎪  Mitotic
ade 4                       ⎪  haploidization
lys 1                       ⎬  data
lys 3                       ⎪
lys 5                       ⎪
ura 1                       ⎪
ura 4                       ⎭
```

Chromosome II

```
ade 7 ⎫ Megnet,
glt   ⎬ personal
leu 1 ⎫ communcation        ⎫
his 7 ⎪                     ⎪
MT    ⎪                     ⎪  Mitotic
his 2 ⎬ Leupold, 1958       ⎪  haploidization
his 5 ⎪                     ⎪  and
leu 3 ⎪                     ⎬  mitotic
met 3 ⎪                     ⎪  crossing-over
ade 1 ⎭                     ⎪  data
arg 4                       ⎪
arg 5                       ⎪
arg 7                       ⎪
lys 4                       ⎪
ura 5                       ⎭
```

Chromosome III

```
Centromere ⎫ Leupold, personal ⎫
ade 6      ⎬   communication    ⎬ Mitotic haploidization data
arg 1      ⎭                    ⎭
```

Chromosome IV
    arg 3   (mitotic haploidization data)

Chromosome V
    arg 6   (mitotic haploidization data)

Chromosome VI
    ade 5   (mitotic haploidization data)

* The cited results by Leupold and Megnet were obtained by meiotic mapping.

This chromosome number, based on genetic evidence, is of course only a minimal estimate. Further mapping of additional markers might reveal some more chromosomes. It is, however, of interest to note that a haploid complement of six chromosomes is in disagreement with the previous cytological studies of Schopfer *et al.* (1963), who had estimated a haploid number of three chromosomes in *Schiz. pombe*. In view of the present genetic evidence, the stained granules observed by those authors seem to be chromosome aggregates, and not individual units.

With the future improvement of cytological techniques the present data may

be confirmed. Presently, however, there is no satisfactory method available to assure an effective chromosome spreading in yeast.

It was not possible, within the limits of the present experiments, to do extensive chromosome mapping by mitotic crossing-over (Pontecorvo, 1958). No direct meiotic linkage had been found, in preliminary attempts (U. Leupold, personal communication), for the 16 new markers located in the present work. Therefore, establishing their order by mitotic crossing-over seems to be the most promising approach.

However, mapping by mitotic crossing-over depends on the availability of appropriate selectors. In *Schiz. pombe*, besides the MT region (iodine treatment), a few other markers may be used as selectors. For example, some genes involved in the biosynthesis of adenylic acid can be useful due to the possibility of colour selection, either directly (*ade 6* and *ade 7*), or indirectly (*ade 1, ade 3, ade 4* and *ade 5*); the latter group of genes blocks the synthesis of adenylic acid at a step earlier than *ade 6* and *ade 7*. Therefore, strains carrying one of those genes in addition to *ade 6* or *ade 7* will form white colonies, whereas *ade 6* and *ade 7* strains will form red colonies.

Among the adenine genes, *ade 1* and *ade 7* are of special interest for mapping genetic markers on chromosome II and *ade 3* can be used as a selector on chromosome I. As the majority of genes are located on these two chromosomes, future efforts towards a more extensive mapping by mitotic crossing-over should be worthy of consideration.

(ii) *pFPA-induced haploidization*

Käfer (1961) has shown that spontaneous haploidization in *Aspergillus* is accomplished by mitotic non-disjunction events resulting in progressive loss of chromosomes in consecutive cell divisions. Lhoas (1961, 1967) suggested that the same mechanism holds true for pFPA-induced haploidization. This hypothesis was confirmed recently (Lhoas, 1968) through cytological studies of hyphal tip cells of *A. niger*.

Mitotic haploidization induced by pFPA in *Saccharomyces cerevisiae* was attempted by Emeis (1966) with little success. Complete haploidization was never achieved, and pFPA treatment of diplonts only induced the formation of unstable aneuploid strains. The first report of effective haploidization of yeast diplonts is that of Gutz (1966) in *Schizosaccharomyces pombe*, and although only one chromosome pair was marked in those early experiments, stable strains monosomic for this chromosome were obtained.

In the present work, experiments involving markers in more than two chromosomes were conducted, and stable haplonts were rescued after treatment with pFPA. This confirms Gutz's findings. However, as Gutz (1966) and Strömnaes (1968) pointed out, it should be stressed here that *Schiz. pombe* is a haploid organism, and thus diplonts might have a strong tendency to re-establish the haploid condition. This would explain the success of pFPA-induced haploidization experiments.

As for the mechanism of action of pFPA, its effects in inducing mitotic haploid-

ization in fungi seem to be closely associated with the inhibitory action of that analogue reported for various organisms (Munier & Cohen, 1959; Martin & Moss, 1949; Halvorson & Spiegelman, 1952). Deficient proteins, resulting from incorporation of pFPA in lieu of phenylalanine, were reported by Kerridge (1960) and Brock (1961). Furthermore, pFPA was found to produce defective mitosis-associated proteins responsible for the delay of cell division, without inhibiting DNA synthesis (Rasmussen & Zeuthen, 1962; Mueller & Kajiwara, 1966). A decrease in the length of the mitotic spindle of human amnion cells in the presence of pFPA was also reported by Sisken & Wilkes (1967), although the precise nature of this phenomenon was not clarified (see Taylor, 1959).

If pFPA does not interfere with chromosome replication (since it does not prevent DNA or protein synthesis), but only with the mitotic process or apparatus, mitotic non-disjunction is likely to occur. It can, thus, be expected that monosomic cells, as well as their complementary polysomic sisters, would be produced. These polysomics (trisomics and above), together with eventual nullisomics, possibly have an unbalanced cell metabolism; they are probably not viable and could account for the many lethal pedigree branches observed in the presence of pFPA. If this is true, the high incidence of lethal pedigree branches seems to agree with the idea that pFPA-induced mitotic haploidization is achieved by chromosome loss in successive mitoses. Because of technical difficulties caused by an increase in division time, the pedigree analyses could not be followed further.

Although these experiments were carried out on a preliminary basis, with a small number of cells, a detailed pedigree analysis in *Schiz. pombe* seems to be a suitable approach towards an understanding of the phenomenon of mitotic haploidization.

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