INDUCTION OF MITOTIC CROSSING OVER IN *SACCHAROMYCES CEREVISIAE* BY BREAKDOWN PRODUCTS OF DIMETHYLNITROSAMINE, DIETHYLNITROSAMINE, 1-NAPHTHYLAMINE AND 2-NAPHTHYLAMINE FORMED BY AN *IN VITRO* HYDROXYLATION SYSTEM

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ABSTRACT

Dimethylnitrosamine and diethylnitrosamine, two potent carcinogens, are nonmutagenic when tested directly in microorganisms. Likewise 1-naphthylamine and 2-naphthylamine are also nonmutagenic but the *N*-hydroxy derivatives are mutagenic in microorganisms. Apparently these compounds require metabolism to breakdown products which are then the proximately active agents, and microorganisms lack the enzymes necessary to effect this conversion. These compounds are mutagenic in Saccharomyces after conversion to breakdown products in an *in vitro* hydroxylation medium. The induction of mitotic crossing over in *Saccharomyces cerevisiae* by breakdown products of dimethylnitrosamine, diethylnitrosamine, 1-naphthylamine and 2-naphthylamine formed in the UDENFRIEND hydroxylation medium is reported in this communication. Mitotic crossing over was detected as red sectored colonies resulting from induced homozygosity of the *ade2* marker. Dimethylamine and diethylamine, which lack the nitroso group of the nitrosamines, did not induce mitotic crossing over under any of the test conditions. To further confirm that the induced sectored colonies were the result of mitotic crossing over they were tested for the presence of reciprocal products. The expected reciprocal products were found in over 67% of the isolates tested. The significance and practicality of using mitotic recombination as an indicator of genetic damage potential of chemicals is discussed.

MITOTIC recombination has been observed in a variety of diploid organisms (STERN 1936; PONTECORVO *et al.* 1953; JAMES and LEE-WHITING 1955; HOLLIDAY 1961; FJELD and STRÖMANES 1965) and probably in mammals as well (GRÜNEBERG 1966; BATEMAN 1967). The potential result of a recombinational event is the generation of a new genotype by crossing over followed by appropriate segregation of the products of mitosis. The consequence to biological systems for such an event is the expression of a detrimental phenotype in the homozygous condition which goes undetected while heterozygous (ZIMMERMAN 1971b). A number of chemicals which are known mutagens and carcinogens cause, in

addition, the induction of mitotic gene conversion (YOST, CHALEFF and FINERTY 1967; ZIMMERMANN and SCHWAIER 1967a; ZIMMERMANN 1968, 1969, 1971a; MARQUARDT *et al.* 1970; PUTRAMENT and BARANOWSKA 1971) and mitotic crossing over (MORPURGO 1963; ZIMMERMANN, SCHWAIER and v. LAER 1966; ZIMMERMANN and SCHWAIER 1967b; ZIMMERMANN and v. LAER 1967). Thus, in a more general way, chemically-induced mitotic recombination serves as an indicator of genetic damage (SIEBERT, ZIMMERMANN and LEMPERLE 1970; ZIMMERMANN 1971a).

Some chemicals are not genetically active themselves but require metabolism to other compounds which are then the proximately active agent. The problem of detecting chemically-induced genetic damage is therefore compounded by differential metabolic treatment of some chemicals by different species of organisms. Dimethylnitrosamine (DMN) is mutagenic in Salmonella auxotroph reversion tests only after metabolism of the parent compound in the host-mediated assay (GABRIDGE and LEGATOR 1969) or by mouse liver microsomes (MALLING 1971), probably to a methylating agent (MAGEE and HULTIN 1962; MAGEE and FARBER 1962; CRADDOCK and MAGEE 1963; LEE, LIJINSKY and MAGEE 1964). The presumed breakdown products of 1-naphthylamine (1-NA) and 2-naphthylamine (2-NA), but not the parent compounds, were mutagenic in *Escherichia coli* auxotroph reversion tests (PEREZ and RADOMSKI 1965; BELMAN *et al.* 1968; MUKAI and TROLL 1969). The above examples are presumably metabolized by a mechanism involving a hydroxylation as the first step (DRUCKREY *et al.* 1961; PREUSSMANN 1964; UEHLEKE 1969). This metabolic step can be mimicked *in vitro* using a hydroxylation medium (PREUSSMANN 1964) developed by UDENFRIEND *et al.* (1954). When DMN and diethylnitrosamine (DEN) are treated in the UDENFRIEND reaction mixture, the resulting breakdown products are mutagenic for Neurospora (MALLING 1966; MALLING and DE SERRES 1972) and Saccharomyces (MAYER 1971). 1-NA and 2-NA breakdown products, formed in the UDENFRIEND reaction mixture, are likewise mutagenic in Saccharomyces (MAYER 1972).

In this communication, the induction of mitotic crossing over in diploid *Saccharomyces cerevisiae* by breakdown products of DMN, DEN, 1-NA, and 2-NA formed in the the UDENFRIEND hydroxylation medium is described.

MATERIALS AND METHODS

Yeast strain: A diploid strain of *Saccharomyces cerevisiae* designated D-3 was kindly donated by DR. F. K. ZIMMERMANN and was previously described (ZIMMERMANN and SCHWAIER 1967b).

Strain D-3 has the genotype:

CYH4	(34)	*ade2*	(38)	*his8*
+		+		+

The numbers in parentheses are the map distances according to a map which was previously described (MORTIMER and HAWTHORNE 1966). The three markers for dominant cycloheximide resistance (*CYH4*), recessive adenine requirement (*ade2*), and recessive histidine requirement (*his8*) are arranged on the same chromosome as shown in the diagram, while the wild-type

alleles are situated on the homologous chromosome. These markers are located on what has formerly been known as Fragment 1. Recent unpublished data of R. K. MORTIMER and D. C. HAWTHORNE indicate linkage of *ade*2 with *pet*17 (genic petite), which in turn is centromere-linked on chromosome XV.

Materials and media: Dimethylnitrosamine (DMN), diethylnitrosamine (DEN), dimethylamine HCl (DMA), and diethylamine HCl (DEA) were obtained from Eastman Organic Chemicals, Rochester, N. Y. At the beginning of each experiment, these four compounds were each dissolved in an aliquot of reaction mixture (see below) to make 100 μmol/ml solutions. 1-Naphthylamine HCl (1-NA) and 2-naphthylamine HCl (2-NA) were obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. 1-NA, because of its greater toxicity, was dissolved in reaction mixture at the beginning of each experiment to make a 0.56 μmol/ml solution while the less toxic 2-NA was employed at 1.34 μmol/ml.

The reaction mixture employed here was developed by UDENFRIEND *et al.* (1954) for the hydroxylation of aromatic compounds. MALLING (1966) modified the concentrations of the components for use with *Neurospora crassa*; the mixture consists of ascorbic acid (20mM), $Fe^{2}+$ as $FeSO_4$ (1mM), and ethylenediaminetetraacetic acid (Na salt) (2mM) dissolved in 1 l. of phosphate buffer at pH 7.4 (0.06M).

Cells were maintained on a semisynthetic complete medium described by PITTMAN *et al.* (1960). A synthetic complete medium was used to test for histidine requirement or cycloheximide resistance. It consisted of a minimal medium (MAGNI and VON BORSTEL 1962) to which was added adenine sulfate, uracil, L-tryptophan, L-histidine HCl, L-arginine HCl, and L-methionine, 20 mg/l.; L-tyrosine, L-leucine, L-isoleucine, and L-lysine HCl, 30 mg/l.; 1-phenylalanine, 50 mg/l.; L-glutamic acid and L-aspartic acid, 100 mg/l.; L-valine, 150 mg/l.; L-threonine, 200 mg/l.; and L-serine, 375 mg/l. For the histidine dependence test, histidine was omitted from the above medium and for the cycloheximide resistance test, cycloheximide was added at 1 μg/ml.

Presporulation medium consisted of 0.8% yeast extract, 0.3% peptone, 10% dextrose, and 2% agar in distilled water. Sporulation medium consisted of 1% potassium acetate, 0.1% yeast extract, 0.05% dextrose, and 2% agar in distilled water.

A snail enzyme preparation (Glusulase, Endo Laboratories, Inc., Garden City, N.Y.) was used to digest ascus walls prior to dissection (JOHNSTON and MORTIMER 1959).

Experimental procedure: For experiments, cells were cultivated on the semisynthetic complete medium for 72 hr at 30°C to achieve a confluent lawn of growth. The cells were rinsed from the agar surface and washed twice in 0.9% saline, and the inoculum was standardized as previously described (MAYER and LEGATOR 1970).

Approximately 5×10^{7} cells/ml were suspended in the reaction mixture and each test compound was added to separate samples at the appropriate concentration. Separate samples were exposed to either oxygen or nitrogen gas bubbled through the reaction mixture containing the cells and compound. Since oxygen is essential for the reaction (UDENFRIEND 1954; PREUSSMANN 1964), the effect of genetically active breakdown products can be measured in samples with oxygen and the effect of reaction mixture and starting compound can be measured in samples exposed to nitrogen. Control samples, consisting of yeast cells suspended in reaction mixture with no test compound, were exposed to oxygen or nitrogen to determine whether the reaction mixture alone had any killing or genetic effects.

DMN, DEN, DMA, and DEA were incubated in the reaction mixture for 6 hr at room temperature. 1-NA and 2-Na were similarly treated for 2 hr. After the treatment period a sample from each flask was appropriately diluted in saline and plated on semisynthetic complete medium (PITTMAN *et al.* 1960).

Detection of mitotic recombination: A homozygous phenotype induced in the heterozygous diploid was the primary criterion for mitotic crossing over. The *ade*2 marker is particularly useful in this respect because the mutant allele results in the accumulation of a red pigment when homozygous but is recessive when heterozygous with the wild type. Thus, mitotic crossing over that results in homozygosity of the *ade*2 marker can be detected as a red sector in a normally white colony.

Appropriate dilutions of cells from the treatment flasks, plated on semisynthetic complete

medium, were incubated for 2 days at 30°C followed by a further holding period of 2 days at 4°C, a procedure which seemed to promote color development and permitted only limited further enlargement of the colonies. Red sectors on plates containing 1,000 to 20,000 colonies were enumerated by using a dissecting microscope at 10× magnification and the surviving population was enumerated on other plates containing approximately 100 colonies per plate.

The appearance of a red sector is not conclusive evidence for the occurrence of mitotic recombination, and further testing is necessary. It is obvious that a mutation at the wild-type *ade*2 allele would result in expression of the recessive phenotype. In addition, gene conversion, aneuploidy, and chromosomal deletions covering the wild-type allele as well as haploidization could also result in expression of the recessive phenotype.

In order to obtain further evidence that mitotic crossing over is the mechanism responsible for the induction of red sectors, an attempt was made to show the reciprocal products from the crossover by using the peripheral markers for cycloheximide sensitivity and histidine requirement. In such instances the red, adenine-requiring clone should also require histidine and will be cycloheximide-resistant. The white clone will be adenine- and histidine-independent like the parental D-3 strain; however, it will be cycloheximide-sensitive if the crossover event occurs between the centromere and the *CYH*4 marker. On the other hand, the white clone will be cycloheximide-resistant like the parental D-3 strain if the crossover event occurs in the region between *CYH*4 and *ade*2.

Red sectors were isolated from DMN, DEN, 1-NA, and 2-NA treatment in reaction mixture and oxygen. In addition, colonies which had fairly large red sectors and which were also well isolated from neighboring colonies were also cloned for the white-sectored portion of the colony. The red clones were tested for histidine requirement and the white clones from each colony containing an *ade*$^-$, *his*$^-$ red sector were tested for cycloheximide resistance. Five white clones from each isolate were tested in an attempt to avoid missing the cycloheximide sensitivity because of delayed segregation of the phenotype. If at least one of the five clones was sensitive to cycloheximide, the isolate was scored as showing both reciprocal products.

A limited sample of cycloheximide-sensitive white clones, which represented the reciprocal product from the crossover event, were used to indicate whether concomitant gene conversion at the *ade*2 or *his*8 locus had occurred. If conversion had occurred then these markers would be heterozygous. This possibility is readily tested by ascus analysis. The white clones were induced to sporulate by cultivating the cells on presporulation medium for 2 days at 30°C followed by cultivation on sporulation medium for 5 days at 30°C. The sporulated cultures were treated with snail enzyme diluted in water (JOHNSTON and MORTIMER 1959) and the asci were dissected according to standard genetic techniques (HAWTHORNE and MORTIMER 1960). The resultant haploid clones from complete asci were scored for adenine requirement by visual observation of red pigmented colonies, and for histidine requirement and cycloheximide resistance on synthetic complete medium as previously described.

Further details concerning this strain for the detection of mitotic crossing over with the *CYH*4, *ade*2, and *his*8 markers were previously described (ZIMMERMANN, SCHWAIER and v. LAER 1966; ZIMMERMANN and SCHWAIER 1967b; ZIMMERMANN and v. LAER 1967).

RESULTS

DMA and DEA, which lack the nitroso group of the corresponding nitrosamines, did not induce mitotic recombination in Saccharomyces (Table 1). The 0 time control contained 72 sectors in a screened population of 58.5×10^4 colonies, resulting in a frequency of 12.3 sectors per 10^5 survivors. Treatment of the yeast cells in the reaction mixture for 6 hr with either oxygen or nitrogen atmosphere resulted in frequencies of 14.2 and 14.7 sectors per 10^5 survivors, respectively. No appreciable increase in the frequency of red sectors was seen with DMA or DEA in the reaction mixture with either oxygen or nitrogen atmosphere. Survival in all samples ranged from 98% to 110% of the 0 time control.

TABLE 1

Lack of induction of mitotic recombination by dimethylamine (DMA) and diethylamine (DEA) in UDENFRIEND'S *hydroxylation mixture in either O_2 or N_2 atmosphere*

Treatment conditions	Percent survival	Population screened	Red sectors	Sectors/10^5 survivors
Control at 0 time	100	58.5×10^4	72	12.3
N_2 atmosphere control	110	45.1×10^4	64	14.2
O_2 atmosphere control	102	41.4×10^4	61	14.7
DMA in N_2 atmosphere	103	28.0×10^4	45	16.1
DMA in O_2 atmosphere	98	27.2×10^4	45	16.6
DEA in N_2 atmosphere	101	28.0×10^4	47	16.8
DEA in O_2 atmosphere	106	29.2×10^4	33	11.3

The results of testing DMN and DEN in the reaction mixture are recorded in Table 2. DMN and DEN in the reaction mixture with nitrogen atmosphere showed no appreciable increase above the 0 time control. With an oxygen atmosphere, however, DMN resulted in a four- to six-fold increase and DEN in a sixty- to eighty-fold increase in the frequency of red sectors among the surviving population. Only the sample from DMN and oxygen in reaction mixture showed a depression in survival.

The results of testing 1-NA and 2-NA for mitotic recombination in yeast cells exposed to the compounds in reaction mixture are recorded in Table 3. The frequency of red sectors in this control sample at 0 time and the sample of 1-NA in

TABLE 2

Induction of mitotic recombination by breakdown products of dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) in UDENFRIEND'S *hydroxylation mixture in an O_2 atmosphere*

Treatment conditions	Percent survival	Population screened	Red sectors	Sectors/10^5 survivors
Control at 0 time	100	58.5×10^4	72	12.3
DMN in N_2 atmosphere	105	36.6×10^4	69	18.4
DMN in O_2 atmosphere	94	32.6×10^4	277	84.4
DEN in N_2 atmosphere	109	38.0×10^4	64	16.9
DEN in O_2 atmosphere	103	26.5×10^4	2707	1020.2

TABLE 3

Induction of mitotic recombination by breakdown products of 1-naphthylamine and 2-naphthylamine formed by UDENFRIEND'S *hydroxylation mixture*

Treatment conditions	Percent survival	Population screened	Red sectors	Sectors/10^5 survivors
Control at 0 time	100	28.5×10^4	34	11.9
1-Naphthylamine in N_2 atmosphere	91	26.0×10^4	30	11.5
1-Naphthylamine in O_2 atmosphere	67	19.2×10^4	73	38.0
2-Naphthylamine in N_2 atmosphere	90	25.7×10^4	39	15.2
2-Naphthylamine in O_2 atmosphere	78	22.1×10^4	68	30.8

TABLE 4

Results of testing red sectors for concomitant histidine requirement

Compound	Red sectors isolated	*his*⁻ Red sectors	*his*⁺ Red sectors
Dimethylnitrosamine	99	96 (97.0%)	3 (3.0%)
Diethylnitrosamine	84	84 (100%)	0 (0%)
1-Naphthylamine	72	66 (91.7%)	6 (8.3%)
2-Naphthylamine	81	80 (98.8%)	1 (1.2%)

TABLE 5

Results of testing reciprocal white clones for cycloheximide sensitivity from colonies containing his⁻ red sectors

Compound	Total white clones tested	Cycloheximide sensitive	Cycloheximide resistant
Dimethylnitrosamine	43	29 (67.4%)	14 (32.6%)
Diethylnitrosamine	44	32 (72.7%)	12 (27.3%)
1-Naphthylamine	24	22 (91.7%)	2 (8.3%)
2-Naphthylamine	22	19 (86.4%)	3 (13.6%)

reaction mixture with nitrogen atmosphere were quite comparable, while the sample containing 2-NA with nitrogen atmosphere was only slightly greater. On the other hand, 1-NA in reaction mixture with oxygen was approximately three times higher than the control, while the same treatment conditions using 2-NA approximately doubled the frequency of red sectors in the population. Survival to naphthylamine treatments was lower (approximately 90%) for nitrogen atmosphere conditions than the 0 time control and was more noticeably lower for oxygen atmosphere conditions, resulting in 67% survival with 1-NA and 78% with 2-NA.

Nearly all of the red sectors isolated required, in addition, histidine (Table 4). Moreover, the majority of the white-sectored portions of colonies containing red, *his*⁻ sectors were found to be cycloheximide-sensitive (Table 5), showing the reciprocal products expected for mitotic crossing over. The results of testing cycloheximide-sensitive white clones for concomitant heterozygosity in the *ade*2 and *his*8 alleles, indicative of gene conversion, are presented in Table 6. In no instance was heterozygosity observed for *ade*2 and *his*8 alleles. Other tests on sporulated

TABLE 6

Phenotype of haploid spores from dissected asci of white, cycloheximide-sensitive clones

Compound	*ade*⁺ Homozygotes	*ade*⁺, *his*⁺ Homozygotes	*ade*⁻ Heterozygotes	*ade*⁺, *his*⁻ Heterozygotes
Dimethylnitrosamine	11/11	9/9	0/11	0/9
Diethylnitrosamine	14/14	8/8	0/14	0/8
1-Naphthylamine	11/11	11/11	0/11	0/11
2-Naphthylamine	10/10	10/10	0/10	0/10

cycloheximide-resistant white clones showed that in each case all three alleles (*CYH4*, *ade2*, and *his8*) were heterozygous like the parental D-3 strain (MAYER, unpublished results) and it was assumed that the reciprocal product of the crossover was not recovered because it seems unlikely that gene conversion could account for heterozygosity in all three genes simultaneously.

DISCUSSION

Mitotic crossing over was induced by DMN, DEN, 1-NA, and 2-NA only when oxygen was bubbled through the hydroxylation medium. No increase in red sector frequency was observed in any samples when nitrogen was substituted for the oxygen atmosphere. Since breakdown in the reaction mixture only occurs in an oxygen atmosphere (UDENFRIEND 1954; PREUSSMANN 1964), the genetic effect observed in such samples must be due to breakdown products and not to the parent compounds themselves. If the parent compounds were genetically active, one would expect to find an increase in the frequency of sectors in the nitrogen samples as well as in the oxygen samples.

The induction of mitotic crossing over by breakdown products of DMN and DEN parallels previous studies on petite mutant and canavanine-resistant mutant induction in Saccharomyces (MAYER 1971) as well as reverse mutation studies in Neurospora (MALLING 1966) in the hydroxylation medium. Similarly, DMA and DEA, which lack the nitroso group of the homologous nitrosamines, did not induce mitotic crossing over as reported here nor were they mutagenic in the hydroxylation medium, as previously reported (MAYER 1971). A certain consistency exists among the data on DMN and DEN in the hydroxylation medium reported by PREUSSMANN (1964), by MALLING (1966), by MAYER (1971), and by this report. DEN breakdown products were more potent mutagens than those of DMN for genic mutation in Saccharomyces (MAYER 1971) and in Neurospora (MALLING 1966), and DEN breakdown products induced a higher frequency of mitotic crossing over than those of DMN as reported herein. PREUSSMANN (1964) previously demonstrated by physical measurement that DEN was 37% metabolized after 6 hr in the UDENFRIEND hydroxylation medium, while DMN was only 20% metabolized. Thus measurements of genic mutation, mitotic crossing over and breakdown are consistent with one another for DMN and DEN.

In a previous report (MAYER 1972), 1-NA and 2-NA breakdown products, formed in the UDENFRIEND hydroxylation medium, induced petite mutants and canavanine-resistant mutants in *S. cerevisiae*. The breakdown products of 1-NA were found to be more potent mutagens than those of 2-NA; this observation is consistent with previous reports (PEREZ and RADOMSKI 1965; BELMAN *et al.* 1968) in which the *N*-hydroxy derivatives were tested directly for mutagenicity. In this communication, mitotic crossing over was induced by 1-NA breakdown products to a slightly higher frequency in the population than those of 2-NA despite the fact that less than half as much 1-NA was used as 2-NA. It is also interesting to note that *N*-hydroxy-1-NA is a more potent carcinogen than is *N*-hydroxy-2-NA (BELMAN *et al.* 1968). Thus the demonstration that 1-NA is

more potent than 2-NA in causing mitotic crossing over after breakdown in the UDENFRIEND hydroxylation medium is consistent with previous results for mutagenicity in the hydroxylation medium (MAYER 1972) as well as with studies of the mutagenic (PEREZ and RADOMSKI 1965; BELMAN *et al.* 1968) and carcinogenic (BELMAN *et al.* 1968) potential of the *N*-hydroxy derivatives tested directly. It seems quite possible that the UDENFRIEND hydroxylation medium is capable of *N*-hydroxylation as well as ring-carbon hydroxylation, and some of the pertinent literature supporting this concept is previously cited (MAYER 1972).

The tests of *ade*$^-$ isolates for concomitant histidine requirement give good presumptive evidence that mutation or gene conversion are not the mechanisms responsible for the red sector induction. A mutation occurring at the *ade*2 locus on the chromosome containing the wild-type allele could give rise to a red sectored colony. Unless there was the rare occurrence of a second mutational event at the *his*8 locus in the same cell, these red clones would be expected to be *his*$^+$. Indeed, a few *ade*$^-$, *his*$^+$ clones were detected (Table 4). The same argument negates the probability that gene conversion is responsible for the *ade*$^-$, *his*$^-$ clones. As is seen in Table 5, the white clones associated with *ade*$^-$, *his*$^-$ sectors, representing the reciprocal product of the crossover event, were found to be cycloheximide-sensitive in 67% or more of the isolates tested. Thus, the expected reciprocal products were obtained for mitotic crossing over's occurring in the region between the centromere and the *CYH4* marker in the majority of the sectored colonies tested.

Measurement of the genetic hazard potential of chemicals has relied heavily on studies of mutation induction, mainly in microorganisms. In addition to mutation, however, other genetic phenomena are of consequence to biological systems. Mitotic recombination (mitotic crossing over and mitotic gene conversion) is a process which leads to somatic variegation, which has potentially detrimental phenotypic properties (ZIMMERMANN 1971b; MORTIMER and MANNEY 1971). Moreover, mitotic recombination apparently occurs at a higher frequency than does mutation in general and might therefore even be of greater importance than mutation. Some of the most practical systems for the detection of point mutations involve the induction of reverse mutation from auxotrophy to prototrophy—for examples, in bacteriophage (DRAKE 1971), bacteria (AMES 1971) and yeast (FINK and LOWENSTEIN 1969; PITTMANN and BRUSICK 1971). All such systems are expected to show a high degree of specificity due to the interaction of the particular genetic lesion in the auxotroph with a specific mutagen. The induction of mitotic recombination by chemicals in Saccharomyces has shown no such specificity (ZIMMERMANN 1971a, 1971b). Finally, there is a good correlation among chemicals which induce mitotic recombination as well as mutation (ZIMMERMAN, SCHWAIER and V. LAER 1966; ZIMMERMANN 1971a, 1971b). Thus, mitotic recombination must be considered as part of a spectrum of genetic effects which indicate the potential of a chemical to cause genetic damage.

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