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Comparison of the Genetic Activity of AF-2 and Nitrofurantoin in Log and Stationary Phase Cells of Saccharomyces cerevisiae

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The effects of nitrofurantoin and AF-2 in stationary phase cells were compared with the effects in log phase cells. The Saccharomyces cerevisiae diploid strain D7 was used. This strain can be used to monitor lethality, gene conversion, mitotic gene recombination, and reversion. Stationary phase cells were not sensitive to nitrofurantoin, but treatment of log phase cells did result in the induction of lethality and gene conversion. Log phase cells were approximately 10 times more sensitive to the lethal effects of AF-2 than were stationary phase cells. The AF-2 induced increase in frequencies of gene conversion and mitotic recombination per surviving cell were similar in both log and stationary phase cells. Gene reversion was induced by AF-2 in stationary cells, but no revertants were induced in log cells. Measurement of nitroreductase activity gave values for log phase cells which were five to sixfold greater than for stationary phase cells. The increased sensitivity of log phase cells to nitrofurans could therefore be in part due to an increased activation of these compounds. It is concluded that the use of log phase cells is optimal for the detection of induced gene conversion and recombination by nitro compounds.

Key words: Saccharomyces cerevisiae, AF-2, nitrofurantoin, nitroreductase, metabolism, mutation

INTRODUCTION

A large variety of chemicals containing a nitro group, for example, derivatives of furans and imidazoles, have been tested and shown to be mutagenic to Escherichia coli and Salmonella typhimurium [13]. The chemicals are not mutagenic per se but are thought to require activation by reduction of the nitro group to the hydroxylamine derivative [12]. The requirement for metabolism to generate active products of these compounds can be demonstrated by the use of nitrofuran resistant strains of bacteria. Such strains are deficient in nitro-reductase activity and are not mutated by nitro compounds [6]. In addition to the metabolic pathway resulting in the formation of electrophiles there exist other alternative pathways of metabolism which can result in deactivation [1].

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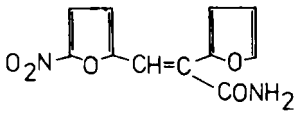
Many nitro compounds have been tested in bacteria but in fungi reports of tests are not as extensive. When tested in Saccharomyces cerevisiae the nitrofuran derivatives AF-2 [3, 9], SQ18506 [7], F30066 [4], a nitroimidazole derivative [2], 2-nitrofluorene, and 2-nitronaphthalene [11] were genetically active to cells harvested from stationary phase cultures when treated for 4 hr or less. However, in some cases genetic activity of nitro compounds could only be demonstrated after treatment using special conditions. For example, niridazole was only positive when growing cells were treated for 24 hr [8], nitrofurantoin, nifurprazium, and FANFT were positive when a respiratory deficient isolate of strain D4 was treated for 16 hr [10]. Metronidazole and two analogues of this compound [2] and 2-nitrobiphenyl [11] were not genetically active in S cerevisiae (treatment times consisted of incubating the test compound with cells from stationary phase cultures for times of 4 hr or less). However, metronidazole has been shown to be mutagenic in the fungus Neurospora crassa if the compound is incubated together with the growing conidia [2].

This investigation was undertaken to determine the basis for the apparent difference in response of fungi, as compared with bacteria, to the genetic and toxic effects of nitro compounds. Two nitrofurans chosen for testing were AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylic acid amide, and nitrofurantoin, N-(5-nitro-2-furfurylidine)-1-aminohydantoin. The structures of these two compounds are in Figure 1.

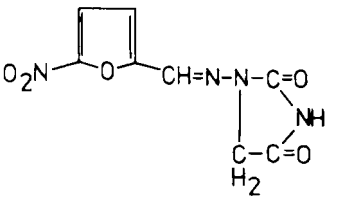
MATERIALS AND METHODS

Strain

D7 has the genotype α/a,ade2-40/ade2-119,trp5-12/trp5-27,ilv1-92/ilv1-92. This multipurpose strain can be used to monitor the frequency of mitotic gene recombination at the *ade2* locus, gene conversion at the *trp5* locus, and gene reversion at the *ilv1* locus [15].



AF-2, 2-(2-FURYL)-3-(5-NITRO-2-FURYL) ACRYLAMIDE



NITROFURANTOIN, N-(5-NITRO-2-FURFURLIDINE)-1- AMINOHYDANTOIN

Fig. 1. Chemical structure of AF-2 and nitrofurantoin.

Treatment Conditions

Cells were harvested from log phase (cell concentration approximately 2×10^7) or stationary phase cultures growing on complete medium (2% peptone, 1% yeast extract, and 2%glucose) and resuspended in 0.1 M phosphate buffer, pH 7.0, at a cell concentration of approximately 3×10^7/ml. Treatments consisted of 5 ml of cell suspension and varying concentrations of the test chemical (dissolved in 1:1 mixture of ethanol and dimethyl sulfoxide). The treatments were shaken aerobically for 60 min at 37°C in the dark and terminated by the addition of ice-cold buffer. The samples were centrifuged, the sedimented cells were washed in buffer, and the cells were finally resuspended in 5 ml of buffer.

Assessment of Genetic Activity

The 0.1 ml aliquots of the washed cells from each treatment were spread on each of five plates of minimal medium without tryptophan to allow estimation of the *trp5* conversion frequency and on each of five plates of minimal medium without isoleucine to allow estimation of *ilv1* reversion frequency. Each treatment was diluted 10^4-fold and 0.1-ml aliquots were spread on each of 30 complete plates to allow estimation of survival, mitotic recombination at the *ade2* locus (red-pink, and red-pink-white colonies) and other genetically altered colonies at the *ade2* locus (red, pink, red-white, and pink-white colonies).

Measurement of Nitroreductase Activity

The reduction of para-nitrobenzoic acid (PNBA) to para-aminobenzoic acid was estimated by the Bratton-Marshall reaction [14]. Cells from log or stationary phase cultures in 2 ml of 0.1 M phosphate buffer at pH7 were incubated with 2.5 mM PNBA for 1 hr at 37°C. Incubations were terminated by adding 2 ml of 6 N HCl and heating to 100°C for 1 hr. The incubations were cooled on ice and to each tube was added 0.5 ml of fresh 0.2% sodium nitrite solution. After mixing and standing for 10 min, 0.5 ml of 1% sodium sulphamate was added, 3 min later 1 ml of 30% sodium acetate and then 0.5 ml of 0.2% N-(1-napthyl)-ethylene diamine HCl was added. Then 10 min were allowed for the reaction to go to completion. The presence of PABA resulted in the formation of a diazotized product which was a purple color. This was extracted with 3 ml of isoamyl alcohol. Centrifugation was usually necessary to facilitate separation of the alcohol layer from cell debri. The alcohol layer was removed and added to 0.1 ml of 25% trichloroacetic acid in ethylene dichloride. The optical density was read at 540 nM on a Ziess, PM2D spectrophotometer.

Chemicals

Nitrofurantoin was purchased from Sigma Chem Company, Missouri, and AF-2 was kindly donated by Dr. J. Kada.

RESULTS

The results of treating strain D7 with various concentrations of nitrofurantoin are given in Figure 2. When stationary phase cells were treated with this compound up to the limit of its solubility in this system (4.2 mM), there was no decrease in cell survival or increase in conversion frequency. However, cells from log

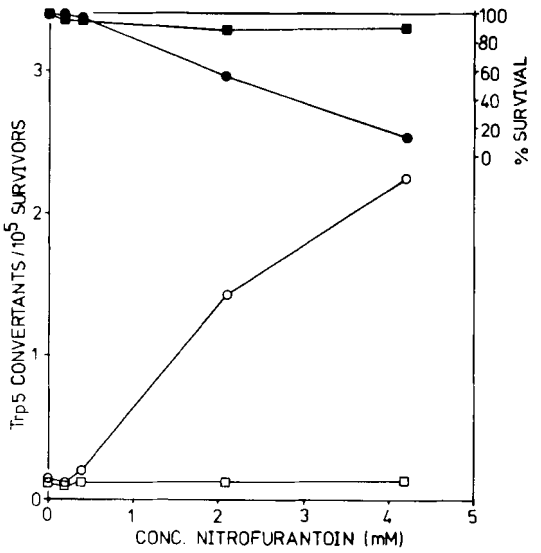


Fig. 2. Survival and induction of gene conversion after treatment of log and stationary phase cells of the yeast strain D7 with nitrofurantoin. Treatments were for 60 min at 37°C in the dark. The survival of log phase cells (●) and stationary phase cells (■) and the conversion frequency of log phase cells (○) and stationary phase cells (□) are given. A duplicate experiment gave similar results.

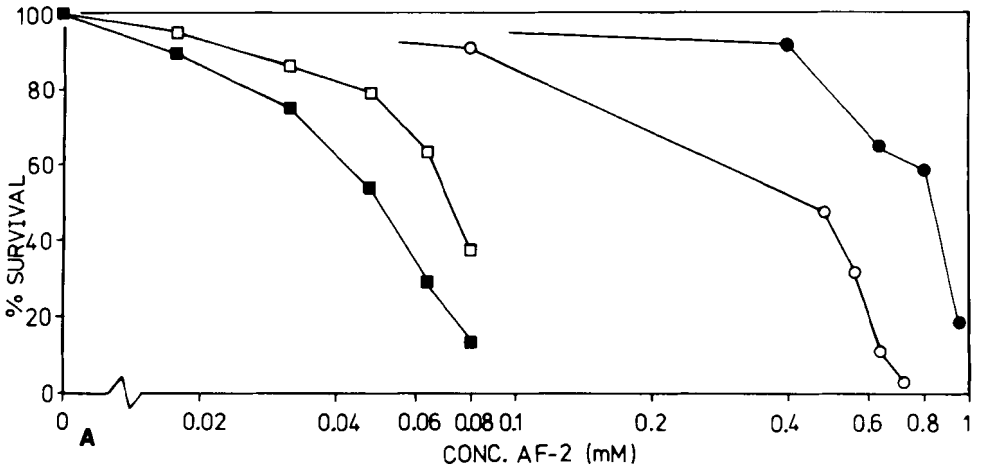
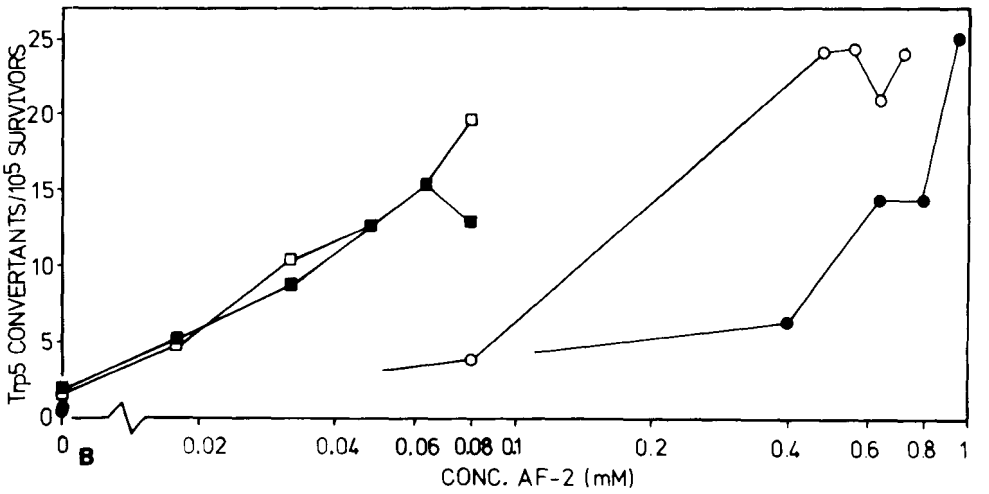
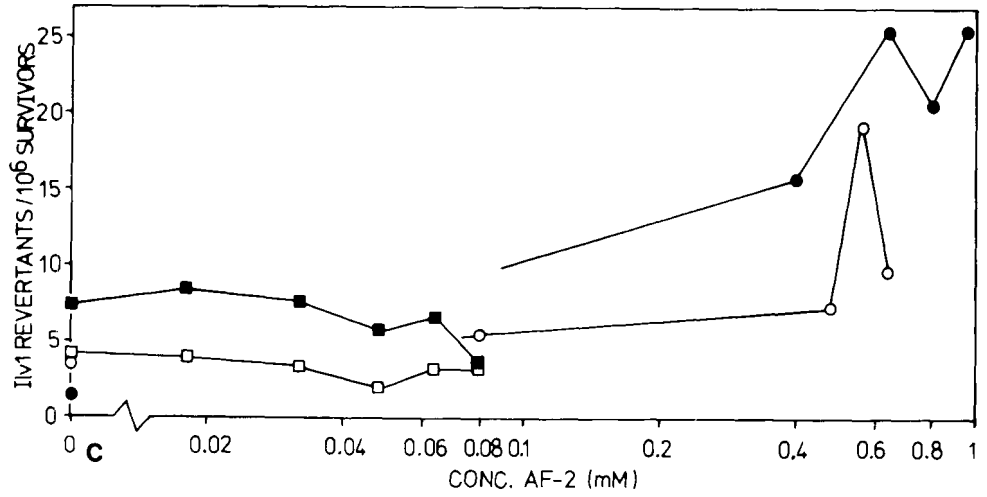
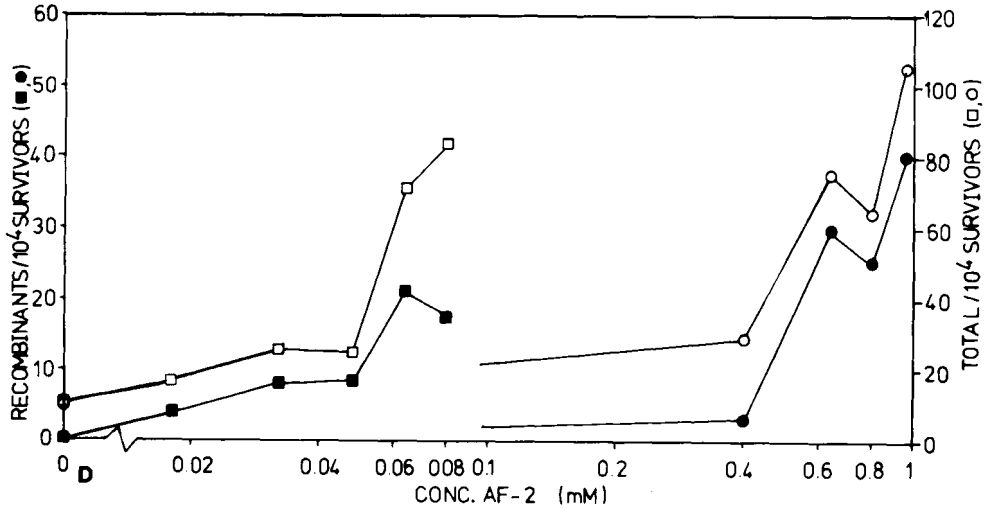


Fig. 3. Survival and induction of gene conversion, mitotic recombination, and reversion after treatment of log and stationary phase cells of the yeast strain D7 with nitrofurantoin. Treatments were for 60 min at 37°C in the dark. (A) Survival of log (■,□) and stationary phase cells (●,○). (B) Induction of gene conversion at the *trp5* locus in log (■,□) and stationary phase cells (●,○). (C) Induction of *ilv1* revertants in log (■,□) and stationary phase cells (●,○). In (A), (B), and (C) the data for two independent experiments are given (represented by the closed and open symbols.) (D) Induction of mitotic recombination at the *ade2* locus. Data presented are the frequency of twin spots in log (■) and stationary phase cells (●) and the total frequency of genetically altered colonies in log (□) and stationary phase cells (○). The data of a single experiment are given (the same experiment is represented by the closed symbols in (A), (B), and (C).


Trp5 CONVERTANTS/10^5 SURVIVORS
0
5
10
15
20
25
B
0
0.02
0.04
0.06
0.08
0.1
0.2
0.4
0.6
0.8
1
CONC. AF-2 (mM)


Ilv1 REVERTANTS/10^6 SURVIVORS
0
5
10
15
20
25
C
0
0.02
0.04
0.06
0.08
0.1
0.2
0.4
0.6
0.8
1
CONC. AF-2 (mM)


RECOMBINANTS/10^4 SURVIVORS (■,●)
TOTAL/10^4 SURVIVORS (□,○)
0
10
20
30
40
50
60
0
20
40
60
80
100
120
D
0
0.02
0.04
0.06
0.08
0.1
0.2
0.4
0.6
0.8
1
CONC. AF-2 (mM)

phase cultures were sensitive to nitrofurantoin, since increasing concentrations of this compound resulted in a decrease in cell survival and an increase in the frequency of *trp5* convertants. The genetic changes at the *ade2* and *ilv1* loci were not estimated.

The results of treating log and stationary phase D7 cells with AF-2 is given in Figure 3. The results from two independent experiments with each cell stage gave similar results. When cells from both stages of growth were treated with increasing concentrations of AF-2 survival decreased. However, the concentration of AF-2 which resulted in, for example, 50% survival of the log phase cells was less (0.05 and 0.072 mM) than the concentration which resulted in a similar survival of the stationary phase cells (0.46 and 0.82 mM). Although the log phase cells responded to a much lower concentration of AF-2 than stationary phase cells the frequency of gene conversion at the *trp5* locus (Fig. 3B), *ade2* mitotic recombinants and frequency of total genetically altered colonies at this locus (Fig. 3D) in log phase cells was similar to that attained in the stationary phase cells. When stationary phase cells were treated with AF-2, there was induction of reversion at the *ilv1* locus (Fig. 3C). However, there was an unexpected absence of induced *ilv1* revertants when log phase cells were treated with AF-2. This was observed in both experiments and for all levels of cell survival.

The nitroreductase activity of yeast cells and of E coli, as estimated from the reduction of para-nitrobenzoic acid is given in Table I. Log phase cells have a nitroreductase activity five - to sixfold greater than stationary phase cells. E coli has an activity similar to yeast when compared on an individual cell basis but when compared on the basis of dry weight it is apparent that E coli possessed an activity about ninefold greater than yeast from log phase cultures.

DISCUSSION

Similar frequencies of cell lethality, gene conversion, and mitotic recombination were induced by AF-2 concentrations which were approximately tenfold less in log as compared with stationary phase cells. When tested in this particular protocol log phase cells were sensitive to nitrofurantoin, whereas stationary phase cells were not. The increased sensitivity of log phase cells to these two nitrofurans may be a general property since reports indicate that two other nitro compounds, niridazole [8] and metronidazole [2], are only genetically active when incubated

TABLE 1. Nitroreductase Activity of Saccharomyces cerevisiae and Escherichia coli

	PABA/10^{10} cells/hr (μmol)	PABA/mg dry wt/hr (μmol)
S cerevisiae, log phase cells	181.8 ± 13.0	0.65 ± 0.05
S cerevisiae, stationary phase cells	29.2 ± 5.0	0.13 ± 0.02
E coli	149.8 ± 18.0	5.70 ± 0.68

Nitroreductase activity was estimated by measuring the reduction of para-nitrobenzoic acid to para-aminobenzoic acid (PABA) (see Materials and Methods). The data are presented as the means of several experiments ± SE. Log phase cells were harvested from cultures at a cell density of approximately 2 × 10^7 cells/ml, stationary phase cells from cultures shaken for 48 hr at 30°C, and E coli from overnight shaken cultures grown in nutrient broth at 37°C.

with growing cells of fungi. Therefore, it is suggested that the use of log phase cells is optimal for the activation of nitro compounds and the detection of the ability of these products to induce gene conversion and recombination. There is an advantage in incubating log phase cells with the test compound in buffer, as compared with growing the cells in the presence of the chemical, since it is possible to estimate cell survival and the possibility of differential growth of mutant and non-mutant phenotypes is eliminated.

The apparent lack of sensitivity of yeast to nitro compounds can be attributed to the low level of nitroreductase activity of these cells as compared with bacteria. Bacteria also possess a higher activity than mammalian tissues. This is the consequence of specific bacterial nitroreductase, whereas in mammalian tissues nitroreductase activity is associated with the enzymes xanthine oxidase, NADPH-cytochrome C-reductase, and aldehyde oxidase [6]. It is possible that yeast is also deficient in specific nitroreductase enzymes. However, the biologically relevant reductases of yeast are unknown and, therefore, it is possible that the enzyme assay used (PNBA reduction) was not assessing all yeast cell nitro-reductase activity.

The difference between the sensitivity of log and stationary phase cells to the two nitrofurans tested may be attributed to difference between the nitroreductase activity of these two cell stages. These differences may reflect differences in enzyme levels. Alternatively, they may arise from differences in intracellular oxygen tensions since anaerobic conditions are known to facilitate the formation of the active product.

The difference in the sensitivity of log and stationary phase cells to AF-2 could also arise from differences in cell permeability or differences in repair. The uptake of the mutagen ethyl methane sulfonate has shown to be greater by log phase cells than by stationary phase cells [5]. It is possible that differential uptake could be contributing to the observed differences in sensitivity of the two cell stages to the nitrofurans. However, although increased uptake and increased nitro-reduction of the nitrofurans in log phase cells would contribute to the greater sensitivity of these cells compared with the stationary cells; these differences do not provide an explanation for the observed absence of gene reversion at the *ilv1* locus in log cells. The reversion frequencies observed at the *ilv1* locus may reflect some differences in repair capabilities between log and stationary phase cells. However, additional experiments using strains with other genetic markers are necessary.

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