EFFECT OF METHYLENE BLUE ON THE ACTION OF 4-NITROQUINOLINE N-OXIDE AND ACRIFLAVINE IN INDUCING RESPIRATION-DEFICIENT MUTANTS OF SACCHAROMYCES CEREVISIAE

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Since a respiration-deficient (RD) mutant of yeast was first obtained by Ephrussi et al. (2) by treatment with acriflavine (AF) and named ‘petite colonie’ mutant, others have been induced by many basic dyes (13), enzyme inhibitors (20), heavy metal salts (8), and physical effects such as ultraviolet irradiation (23) and heat shock (27).

In previous papers (9, 10) we reported that a carcinogenic agent, 4-nitroquinoline N-oxide (4-NQO), could induce an RD mutant which was seemingly like the mutant induced by AF. From the viewpoint of induction rate and the time required for the induction of RD mutants, the effects of 4-NQO and AF were unlike. The mutants produced by these two agents differed in their cytochrome content and in enzyme activities such as catalase, succinic dehydrogenase, lactic dehydrogenase, etc. (12). Consequently, it was thought that the mechanisms of the induction of RD mutants by both agents might be different.

Extensive studies (7, 14, 15, 17, 24) on the mechanism of the induction of RD mutants with AF have been made which suggest the elimination of cytoplasmic elements and the change of nucleic acid base components as the action of AF. Nagai (15) found that the induction of RD mutants by AF was counteracted by methylene blue and toluidine blue, and from his results he suggested that AF exerted its RD-inducing action in a certain specific mode of binding between the dye molecules and receptor material of yeast cells.

The present paper deals with the difference in the effect of methylene blue on the induction of RD mutants by 4-NQO and AF.

MATERIALS AND METHODS

The strain of diploid Saccharomyces cerevisiae (Hansen 0209), obtained from the Institute for Fermentation, Osaka, was used.

Ogur’s glucose medium (19) containing polypeptone 3.5 g, yeast extract 3.0 g, KH₂PO₄ 2.0 g, MgSO₄·7H₂O 1.0 g, (NH₄)₂SO₄ 1.0 g, glucose 10.0 g and agar 15.0 g per 1000 ml of distilled water was used for selection and cultivation of the yeast.

Methylene blue (MB) was obtained from Merck, Darmstadt; acriflavine (AF) from Takeda Co., Osaka; and 4-nitroquinoline N-oxide (4-NQO) from Dr. Eisaku Hayashi,
Shizuoka College of Pharmacy.

The yeast cell suspension was prepared by harvesting the growth from 18 hours old cultures, washing three times with distilled water and resuspending in M/15 Sørensen's phosphate buffer at pH 7.0. This cell suspension was shaken in L-type tube for 16-18 hours at 30°C to obtain the resting cells. The cells were collected by centrifugation, washed twice with distilled water and resuspended in M/15 phosphate buffer at a concentration of 1 mg cells/ml (dry weight) photometrically. The resting yeast cells were exposed to each agent for specified periods at 30°C with occasional stirring, collected by centrifugation and washed twice with distilled water. The treated yeast cells were diluted appropriately, spread on Ogur's agar plates and incubated for 48 hours at 30°C. Detection of RD mutants was done by Nagai's 2,3,5-triphenyltetrazolium chloride (TTC) overlaying method(13) and the rate of induction of RD mutants was calculated against the total colonies growing on the plates.

RESULTS

The effect of pH on the induction of RD mutants of the yeast treated with MB

The resting cells of the yeast were treated with 30 μg/ml of MB at pH 5.0, 7.0 and 9.0 for 3 hours at 30°C. At pH 5.0 and 7.0, few or none RD mutants were induced and at pH 9.0, up to 3.5% of RD mutants were induced (Table 1). Since the induction of RD mutants by 4-NQO and AF was effective in neutral or alkaline buffer solutions, M/15 Sørensen's phosphate buffer at pH 7.0 was used in all experiments.

Table 1. The rate of appearance of RD mutants of the yeast after the treatment with MB at different pH values

<table>
<thead>
<tr>
<th>M/15 Phosphate buffer at pH</th>
<th>Experiments</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>plus 30 μg/ml MB</td>
<td>%</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>0.0</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>0.0</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>0.0</td>
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</tbody>
</table>

The resting cells of Saccharomyces cerevisiae were treated with 30 μg/ml of methylene blue (MB) at different pH values for 3 hours at 30°C. Respiration-deficient (RD) mutant was detected by TTC overlaying method(13). The numerals in the table indicate the rate of appearance (%) of RD mutants calculated against the total colonies growing on Ogur's agar plates(19) after 2 days incubation at 30°C.

* MB solution which was stored at 4°C for 5 days in the dark was used.
Spontaneous RD mutation of the yeast usually occurred at a rate of less than 0.01%, it was not considered as the cause of induction mentioned above.

When MB solution stored at 4°C or room temperature for a few days was used, the induction rate of RD mutants was raised up to 3% even at pH 7.0 (Table 1). No differences were found between stored and freshly prepared MB solutions in their behaviour on paper chromatography and ultraviolet absorption spectra. The cause of the high induction rate by aged MB is now being studied.

The effect of MB on the induction of RD mutants of the yeast treated with 4-NQO and AF

Resting yeast cells were treated with 4-NQO or AF mixed with MB for 3 hours at 30°C in M/15 phosphate buffer at pH 7.0. 10⁻⁶M (ca. 0.2 μg/ml) of 4-NQO, 2.5 × 10⁻³M (ca. 7.5 μg/ml) of AF and 10, 20 and 30 μg/ml of MB in final concentration were used, respectively. As seen in Table 2, the induction of RD mutants by 4-NQO remained unchanged while that of AF had a tendency to be diminished.

As reported in previous papers⁹,¹⁰, the induction rate of RD mutants by 4-NQO approached the maximum after 2 to 4 hours treatment, but it required at least 20 hours treatment with AF to attain the maximum induction rate of RD mutants. Consequently, resting yeast cells were treated with AF mixed with MB for 20 hours. Under these conditions the control system, treated only with MB, produced 3.7% RD mutants. Whether the induction of RD mutants by MB was caused by MB itself or by degradation products of MB is still uncertain.
The effect of 4-NQO and AF on the induction of RD mutants of the yeast pretreated with MB

Resting yeast cells were pretreated with 10, 20 and 30 µg/ml of MB for 3 hours at 30°C in M/15 phosphate buffer at pH 7.0, washed three times with distilled water and then exposed to 10⁻⁶ M 4-NQO for 3 hours or to 2.5×10⁻⁵ M AF for 20 hours. As seen in Table 3, the induction of RD mutants by AF was apparently suppressed by pretreatment with MB, going as high as 60% following pretreatment with 30 µg/ml of MB. These results agreed fairly well with those reported for growing yeast cells by Nagai(15). On the contrary, RD mutation by 4-NQO was enhanced rather than suppressed as a result of pretreatment with MB. No RD mutants were induced in the control system in which resting yeast cells were treated with MB, washed three times with distilled water and incubated in M/15 phosphate buffer for 20 hours at 30°C.

Table 3. The effect of 4-NQO and AF on the induction rate of RD mutants of the yeast pretreated with MB

<table>
<thead>
<tr>
<th>Pretreatment with MB</th>
<th>Further treatment with mutagen</th>
<th>Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻⁶M 4-NQO</td>
<td>1</td>
</tr>
<tr>
<td>—</td>
<td></td>
<td>2.8</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>&quot;</td>
<td>3.0</td>
</tr>
<tr>
<td>20 µg/ml</td>
<td>&quot;</td>
<td>2.9</td>
</tr>
<tr>
<td>30 µg/ml</td>
<td>&quot;</td>
<td>3.3</td>
</tr>
<tr>
<td>—</td>
<td>2.5×10⁻⁵M AF</td>
<td>15.3</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>&quot;</td>
<td>6.3</td>
</tr>
<tr>
<td>20 µg/ml</td>
<td>&quot;</td>
<td>6.4</td>
</tr>
<tr>
<td>30 /ml µg</td>
<td>&quot;</td>
<td>6.4</td>
</tr>
<tr>
<td>10 /ml /µg</td>
<td>None</td>
<td>0.0</td>
</tr>
<tr>
<td>20 /µg /ml</td>
<td>&quot;</td>
<td>0.0</td>
</tr>
<tr>
<td>30 /µg /ml</td>
<td>&quot;</td>
<td>0.0</td>
</tr>
<tr>
<td>Control (Phosphate buffer only)</td>
<td></td>
<td>0.0</td>
</tr>
</tbody>
</table>

The resting cells of the yeast were pretreated with methylene blue (MB) for 3 hours at 30°C in M/15 phosphate buffer at pH 7.0, washed 3 times with distilled water and exposed to 10⁻⁶M 4-nitroquinoline N-oxide (4-NQO) for 3 hours and to 2.5×10⁻⁵M acriflavine (AF) for 20 hours. Other conditions as in Table 1.

DISCUSSION

While MB itself induced almost no RD mutants of the yeast under our experimental conditions, it did reduce the effect of AF in inducing RD mutants of the yeast but showed no effect on the action of 4-NQO. Since MB, at the concentrations used in the experiment, did not suppress the growth of RD mutants induced by 4-NQO and AF, it might not be selective against both RD mutants.
It was more effective to expose the yeast cells, which were pretreated with MB, against AF, rather than to expose them to AF mixed with MB. As for the effect of MB on AF in inducing RD mutants, Nagai\textsuperscript{(15)} stated, “The most probable interpretation is that MB actually binds to a certain cellular material of the yeast and blocks the access of AF to a receptor site which is responsible for the production of RD mutant progeny.” This interpretation is interesting because of the similarities of the molecular structures of AF and MB.

Many studies have been carried out on the mechanisms of the induction of RD mutants by AF\textsuperscript{(7,14,15,17,24)}, and the possibilities may be summarized as the alteration of base-pairing in DNA and as the elimination or diminution of cytoplasmic elements due to the action of AF. Though it was not precise in the yeast, Brenner et al.\textsuperscript{(1)} reported that acridines acted as mutagens because they caused insertion or deletion of base-pairs in DNA of T4 bacteriophage. Lerman\textsuperscript{(7)} also suggested that combination of DNA with acridines resulted in a complex, changing physical characteristics of the DNA. It may be possible that the same phenomena occur in the yeast and that nonmutagenic MB protects the nucleic acid chain from the intercalation of mutagenic AF.

The effect of AF on cytoplasmic elements were observed in the kinetoplast in trypanosomes\textsuperscript{(6)}, R-factor in Enterobacteriaceae\textsuperscript{(11,25)} and F-factor in Escherichia coli\textsuperscript{(5)}. In the last case, the effect of AF in eliminating F-factors was counteracted by MB\textsuperscript{(4)}. Recently, Sugimura\textsuperscript{(24)} assumed that AF suppressed the replication of cytoplasmic particles\textsuperscript{(6)} which were required for the maintenance of the respiratory activities of the yeast. Since the induction of RD mutants by AF was suppressed by MB, this assumption may also hold true in explaining the protective effect of MB against AF elimination of the F-factor in E. coli.

On the other hand, since the induction of RD mutants by 4-NQO was not suppressed by MB, it was thought that the action of 4-NQO on the yeast in inducing RD mutants might differ from that of AF. Okabayashi\textsuperscript{(20)} assumed that 4-NQO induced mutants of Aspergillus niger because of the substitution reaction of 4-NQO with -SH containing compounds in the fungus and the consequent production of another mutagen, nitrous acid. Recently Okabayashi\textsuperscript{(21)} reported that 4-hydroxyaminoquinoline N-oxide (4-HAQO), a reduced metabolite of 4-NQO, was effective as mutagen. This finding is interesting in connection with Ono’s report\textsuperscript{(23)} who showed that 4-HAQO lowered the transforming activity of DNA while 4-NQO could not do so. On the contrary, Nagata et al.\textsuperscript{(18)} attributed the efficiency of 4-NQO to the charge transfer phenomenon. In addition, in regard to the induction of RD mutants of the yeast, 4-NQO was more effective than 4-HAQO. Therefore, further studies will be needed to establish the mutagenic effects of 4-NQO and 4-HAQO.

When the TTC overlaying method\textsuperscript{(13)} was used for the detection of RD mutants, colonies of the yeast treated with AF coloured red (normal) and white (RD), while ones treated with 4-NQO produced pink (intermediate), sectoral and deep red (respiration-sufficient?) colonies in addition to normal and RD colonies. Since this variety of colonies resembled those of ultraviolet irradiated yeasts\textsuperscript{(17,23)}, the possibility exists that RD mutants induced by 4-NQO include cytoplasmic and nuclear gene
As mentioned above, the most probable interpretation for the induction of RD mutants was that AF eliminated or decreased the cytoplasmic elements which were required for the maintenance of the respiratory activities of the yeast and that MB got in the way of AF. However, the mechanisms of AF affecting the cytoplasmic elements are still obscure. On the other hand, the induction of RD mutants by 4-NQO was caused rather by the change in DNA base composition. It is interesting in connection with this that guanine-cytosine contents of DNA in RD mutants of various groups of micro-organisms induced by 5-fluorouracil, 5-bromouracil and copper treatments were higher than those of parental DNA (3). From the fact that cytoplasmic elements such as F-factor and R-factor consist of DNA, it can not be said that 4-NQO has no effect on the cytoplasmic elements. Further studies will be needed to establish differences between mutagenic action of 4-NQO and that of AF on the yeast.

SUMMARY

Methylene blue which was almost ineffective in the production of respiration-deficient mutants of Saccharomyces cerevisiae suppressed the induction of the same mutants by acriflavine but did not show any effect on the mutation induced by 4-nitroquinoline N-oxide.

The authors are grateful to Dr. Eisaku Hayashi of Shizuoka College of Pharmacy in providing 4-NQO. Thanks are due to Miss Setsuko Kurita for her cooperation in this work.

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EFFECT OF METHYLENE BLUE ON RD MUTATION


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(24) Sugimura, T. Personal communication.

