36. Tadashi Okabayashi, Akihiro Yoshimoto, and Misao Ide:
Mutagenic Activity of 4-Hydroxyaminoquinoline 1-Oxide.

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Much interest has been paid for 4-nitroquinoline 1-oxide (4NQO) and its related nitro compounds, since they have outstanding mutagenic activity as well as carcinogenic activity.1-6) As the most widely accepted hypothesis for the action mechanism of these compounds, up to quite recently, it was assumed that the substitution of nitro group at position 4 of 4NQO with sulfhydryl group of living materials and liberation of nitrous acid (reaction (1) in Chart 1) is the primary and most important step.1,5,6) It should be noted, however, that there has been no experimental evidence to connect directly this substitution reaction with mutagenic or carcinogenic action.

\[
\begin{align*}
\text{NO}_2 & + \text{RSH} \rightarrow \text{S}^\text{+} \text{N}_2 \text{H}_5 \text{O} + \text{HNO}_2 \\
\text{NO}_2 & \rightarrow \text{NHOH} + \text{NH}_2 + \text{NH}_2
\end{align*}
\]

Chart 1.

Recently we demonstrated that the nitro group of 4NQO is reduced very rapidly by the suspension of various microorganisms.7) Furthermore, accumulation of large amount of 4-hydroxyaminoquinoline 1-oxide (4HAQO) was also demonstrated after the exposure of 4NQO to Aspergillus niger which is readily mutated by 4NQO. These facts are rather unexpected if we assume that the substitution reaction is the primary reaction of 4NQO with biological materials.

*1 Fukushima-ku, Osaka (岡林 直, 吉本明弘, 井手 篤).
7) T. Okabayashi, A. Yoshimoto : This Bulletin, 10, 1221 (1962); Ibid., 12, 262 (1964).
In this paper, the results of the study about the mutagenic activity of 4HAQO will be given. A preliminary account of this work has already been reported.\(^3\)

**Materials and Methods**

**Microbial Strain**—A laboratory strain of *Aspergillus niger*, *A. niger* W, was used throughout this work. This strain is the same as that previously used for the demonstration of mutagenic activity of 4NQO.\(^1\)

**Culture Media**—In usual experiments following medium (designated as medium A) was used: 50 g. of glucose, 5 g. of bactopeptone, 2 g. of yeast extract, 1 g. of K$_2$HPO$_4$, 1000 ml. of H$_2$O. For the detection of auxotrophs glucose–Capek’s medium was used as a minimal medium.

**Preparation of Conidial Suspension**—*A. niger* W was incubated on medium A agar slope for 4 days at 28°. The conidiospores present were brushed into 10 ml. of sterile H$_2$O and shaken vigorously for 10 min., after which the suspension was filtered through a sintered glass funnel (Ishii No. 2).

**Treatment of 4HAQO**—To 8.5 ml. of 0.1% NaCl solution containing 0.1% KH$_2$PO$_4$ was added 1 ml. of spore suspension containing about 1 x 10$^6$ conidiospores. The suspension was then added with 0.5 ml. of 75% EtOH solutions containing 125 to 1000 μg. of 4HAQO-HCl.\(^2\) The final pH of the suspension was 5.6. The suspension was allowed to stand at 28° for 24 hr., after which aliquots of decimal dilutions were plated on medium A agar. Untreated control was made by the same manner as described above except that 0.5 ml. of 75% EtOH solution was added in place of 4HAQO-HCl solution. After the 72 hr. incubation at 28°, the appeared colonies were picked up at random and incubated on medium A agar for 1 week. Then the morphological properties of isolated progenies were tested. As described later the nutritional requirements of progenies were also examined.

Plating were made in sets of 10 or 20 plates for each dilution. The frequencies of survivors with or without treatment were calculated from the number of colonies on medium A agar plates and from the dilution ratio. The surviving conidiospores of *A. niger* decreased considerably, when they were incubated in NaCl + phosphate solution at 28° for 24 hr. However no special attention was paid for this autodecay phenomenon.

**Paper Chromatography**—Paper chromatography was performed by use of methanol heptane as the solvent. This solvent system is suitable for the separation and detection of nitroquinoline 1-oxides.\(^3\)

**Results**

**Preliminary Experiment on the Survival Ratio and Mutagenicity**

Table I shows the results obtained in a preliminary experiment. The survival ratio of conidiospores after treatment with 4HAQO varied considerably from experiment to experiments. Therefore the data indicated in the table does merely illustrate the tendency on the spore cidal action of 4HAQO. However, it may be regarded that spore cidal action of 4HAQO is 1/30 to 1/100 as active as that of 4NQO. The weakness of spore cidal action of 4HAQO may be explained by low permeability of this reagent in the spores due to its low lipophilic character and the chemical instability of this compound especially to air oxidation.

Alternatively, the table shows that considerable number of morphological mutants can be obtained when survival ratio decreased below 34%.

<table>
<thead>
<tr>
<th>4HAQO (μg./ml)</th>
<th>0</th>
<th>12.5</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival per cent</td>
<td>100</td>
<td>100</td>
<td>66.5</td>
<td>34.1</td>
<td>17.7</td>
</tr>
<tr>
<td>Number isolated</td>
<td>323</td>
<td>180</td>
<td>112</td>
<td>236</td>
<td>197</td>
</tr>
<tr>
<td>Morphological mutants obtained</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>25</td>
<td>26</td>
</tr>
</tbody>
</table>

\(^2\) Kindly supplied by Prof. Masakazu Hamana, of Kyushu University and Dr. Ryözo Maeda of our laboratory.

8) T. Okabayashi: This Bulletin, 10, 1127 (1962).
The Isolation of Mutants

To obtain information on the properties of mutants, nine independent experiments were carried out in the same condition for 4HAQO treatment. In these experiments conidiospores of A. niger were exposed to 100 μg./ml. of 4HAQO-HCl. Survival ratios fluctuated from 64.5% to 98.4% (average 89.2%). After random isolation of 1271 progenies 443 mutants (34.1% of total isolated strains) were obtained. These are summarized in Table II. These results coincide, at least qualitatively, with our previous work performed by use of 4NQO and the same organism. It is interesting to see that sclerotia type variants, the formation of which by induced mutation has rarely been reported, were also obtained in the present experiment as in the case of 4NQO treatment.19

Table II. Mutants Obtained after Treatment of 4HAQO

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Number isolated</th>
<th>Mutants</th>
<th>Number isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light type</td>
<td>121</td>
<td>yeast type</td>
<td>1</td>
</tr>
<tr>
<td>Restricted type</td>
<td>114</td>
<td>sclerotia type</td>
<td>4</td>
</tr>
<tr>
<td>Extremely restricted type</td>
<td>15</td>
<td>brown type</td>
<td>17</td>
</tr>
<tr>
<td>Sterile type</td>
<td>24</td>
<td>olive type</td>
<td>5</td>
</tr>
<tr>
<td>Small head type</td>
<td>7</td>
<td>mycelium yellow type</td>
<td>8</td>
</tr>
<tr>
<td>Chromogenic type&lt;sup&gt;13&lt;/sup&gt;</td>
<td>19</td>
<td>auxorhops</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>intermediate type</td>
<td>96</td>
</tr>
</tbody>
</table>

<sup>a</sup> We tentatively refer to the mutants as chromogenic mutants which secrete coloring material(s) in the medium.

Nutritional Requirements of Auxotrophs

The selection of auxotrophic mutants was carried out by transfer of isolated progenies to Czapek's medium. The strains which failed to grow on Czapek's medium were tested for their nutritional requirements. Among 12 strains one could grow by the external supply of vitamin mixture, and another one could grow by the addition of nucleobases. The remainder (10 strains) grow on a medium supplemented with Casamin Acids plus cystine, glycine and tryptophan. The amino acids requirements of these strains were determined by auxanography. Six strains were found to be arginine auxotrophs, and one was methionineless mutant. The rest (3 strains), which could grow by the addition of either cystine or methionine, could also grow on Czapek's medium supplemented with Na<sub>2</sub>S<sub>O<sub>3</sub></sub>. Thus the strains may be included in reduced sulfur mutants.19

Stability of Mutants

All mutants isolated were transferred successively on medium A agar for five generations. Considerable mutants performed reverse mutations, but more than half of these mutants retained the original properties.

Possibility of Contamination of 4NQO in the Preparation of 4HAQO

As described above, the spore cidal activity of 4HAQO is extremely smaller than that of 4NQO. Therefore we must pay attention to the possibility that the results indicated in this experiment might be due to the contamination of small amount (about 1%) of 4NQO. To rule out this possibility we performed the recrystallization of 4HAQO-HCl. 4HAQO-HCl (21.3 mg.) were dissolved in a small volume of warm methanol and precipitated by the addition of ethyl acetate. After repetition of this procedure for

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three times the precipitate was vacuum-dried (16.5 mg.). The supernatants were collected, evaporated to dryness and dried (6 mg.). If our sample was contaminated by 4NQO it must be concentrated in the supernatant fraction because of its higher solubility in ethyl acetate. Examination of sporeidal and mutagenic activities revealed that the precipitate retained both activities as active as those of original preparation. The supernatant fraction did not show the activities so strong as those expected when we assume that our original 4HAQO sample is contaminated with NQO. Fig. 1 shows the paper chromatogram of each fraction. No detectable amount of 4NQO was present in both fractions. Above facts may exclude the possibility that mutagenic activity of our preparation is due to the contamination of 4NQO.

**Discussion**

It has been reported that 4NQO is one of the most powerful chemical mutagens on *A. niger*,<sup>15</sup> *A. oryzae*<sup>13</sup> and *Streptomyces griseoflavus*,<sup>9</sup> while this reagent has only a weak mutagenic activity on *Escherichia coli*.<sup>19</sup> The choice of *A. niger* in the present experiment is based on the fact that this organism is most sensitive to 4NQO and very easily mutated by this reagent.

Although the present experiment did give only qualitative results, it can be considered that 4HAQO has similar activity to that of 4NQO with regard to the mutagenic activity. Therefore the biological reduction of 4NQO and the subsequent accumulation of 4HAQO (equation 2 in Chart 1) may have very important roles from the viewpoint of mechanism of mutagenic activity of 4NQO. Very recently Shirasu and Ohta demonstrated that 4HAQO is carcinogenic on mice.<sup>19</sup> It was also reported by Endo that 4HAQO caused the bacteriophage induction in lysogenic bacteria and the formation of the characteristic intranuclear inclusions in tissue culture cells.<sup>19</sup> These findings also suggest that 4HAQO is the proximate carcinogenic or mutagenic substance.

The significance of hydroxylamin compounds in the toxicity of drugs was first demonstrated by Chanon, Mills and Williams, who demonstrated that 4-hydroxyamin-2,6-dinitrotoluene was the proximate toxic substance in the case of administration of 2,4,6-trinitrotoluene to rabbits.<sup>15</sup> The significance of their work has been overlooked for about twenty years. Our present work and the finding of Shirasu and Ohta, and Endo may imply that the biological reduction of nitro compounds plays important roles also in mutagenicity or carcinogenicity. In this respect it is interesting to consider the recent studies of Cramer, Miller and Miller,<sup>16</sup> and Troll and Nelson,<sup>17</sup> who demonstrated the formation of N-hydroxylated compounds followed the administration of carcinogenic amines such as 2-acetamidofluorene and 2-naphthylamine to animals.

14) H. Endo : Private communication.
studies also suggested that N-hydroxyamino compounds may be possible carcinogenic substances. The roles of hydroxyamino compounds in the metabolism of drugs will be a fascinating problem in future studies.

In 1955 one of us (Okabayashi) suggested that the substitution reaction of nitro group of 4NQO with sulfhydryl compound(s) and the subsequent liberation of nitrous acid (reaction (I) in Chart 1) may contribute to mutagenic action. Later the speculation was partly adopted by Nakahara and his group for the postulation of carcinogenicity of 4NQO. Since these postulations based on equation (I) are not contradictory to the current concept of molecular genetics, we cannot rule out completely the possibility that reaction (I) has relation to carcinogenic or mutagenic activity. In this respect the finding of Hayashi is important. He demonstrated that the painting of 4NQO on a mouse skin caused the rapid decrease in interepidermal mercapto content. Later this was confirmed by Takayama and Oota showing that there exists a distinct correlation between the histochemical localization of mercapto compound(s) and the localization of autoradiographic grains in the skin treated with tritium labeled 4NQO. These facts may be regarded to be available experimental proofs that reaction (I) really occurs in biological materials. It should be noted, however, that although these findings explain the mechanism for the vesicant action to the skin, one of the most characteristic properties of 4NQO, there is no proof that the mechanism for the vesicant action to the skin is the same as that for mutagenicity or carcinogenicity.

Recently Hayashi informed us that 4HAQO lacks the vesicant action to the skin. This might mean that this action can be separable from that of mutagenicity or carcinogenicity.

We wish to thank Prof. Emeritus Eiji Ochiai of Tokyo University for encouragements and advice throughout this work. We also thank Miss Miyuki Ōsubo for her technical assistance. The work presented here was carried out by a Grant-in-Aid from the Ministry of Education.

Summary

Mutagenic activity of 4-hydroxyaminoquinoline 1-oxide was demonstrated by using a strain of Aspergillus niger as a test organism. The significance of the present experiment is discussed in relation to the mechanism of biological activities of 4-nitroquinoline 1-oxide.

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20) Y. Hayashi: Private communication.