Oxidation of Sulphydryl Compounds in Vitro by 4-Hydroxyaminoquinoline-1-oxide, a Carcinogenic Metabolite of 4-Nitroquinoline-1-oxide

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SUMMARY

Glutathione and cysteine were found to be markedly oxidized by 4-hydroxyaminoquinoline-1-oxide in vitro at pH 7.0 at 37°C, but not at all by 4-aminoquinoline-1-oxide. The sulphydryl compounds were oxidized by 4-hydroxyaminoquinoline-1-oxide to a much smaller extent in the absence of oxygen than in its presence. No substitution reaction between the sulphydryl compounds and 4-hydroxyaminoquinoline-1-oxide was detected, nor any other alteration in the 4-hydroxyaminoquinoline-1-oxide. Regardless of the concentration of 4-hydroxyaminoquinoline-1-oxide used, oxidation followed first-order kinetics, indicating that the role played by the 4-hydroxyaminoquinoline-1-oxide is purely catalytic. Presumably, the reaction mechanism involves the formation of free radicals from 4-hydroxyaminoquinoline-1-oxide. The greater carcinogenic potency of 4-hydroxyaminoquinoline-1-oxide in comparison with 4-nitroquinoline-1-oxide may be correlated with the difference in the mode of reaction with sulphydryl compounds.

INTRODUCTION

For nearly a decade since the potent carcinogenicity of 4NQO was first reported by Nakahara et al. (19), the ability of this substance to produce tumors has been repeatedly demonstrated in various organs and tissues. The mechanism of the production of tumors by this substance has been extensively studied by a number of investigators (1, 4, 9-14, 16, 21, 26, 31, 32) with many interesting results. Of special interest is the substitution reaction of the nitro group of 4NQO with sulphydryl compounds. This reaction takes place most readily, without enzymatic intervention, in a physiological pH range, and none of the reaction products (nitrous acid and S-substituted compounds) have been found to be carcinogenic (18, 21). The reactivity of various quinoline derivatives with the sulphydryl group is definitely correlated with the carcinogenicity of those derivatives in vivo (7) as well as in vitro (3, 25). Accordingly, it has been assumed that the carcinogenicity may in some way be dependent on the substitution reaction of those derivatives with sulphydryl compounds.

Nevertheless, 4NQO was recently found to be metabolically converted by some microbes (23, 24) and rat tissues (8, 29, 30) to 4HAQO through 4AQO. Of the two metabolites, only 4HAQO was shown to be carcinogenic in mice (28) and rats (5). In fact, 4HAQO has since been reported to be definitely more carcinogenic than 4NQO (6, 27). These findings suggest that 4NQO acts carcinogenically in the form of its partially reduced metabolite.

Although sulphydryl compounds have been found to react with 4NQO and certain of its carcinogenic derivatives, it has not previously been demonstrated whether sulphydryl compounds react with 4HAQO and 4AQO. The present study was designed to elucidate the nature of any interaction between sulphydryl compounds and these reduced derivatives of 4NQO in vitro.

MATERIALS AND METHODS

Preparation of 4HAQO. 4HAQO was prepared by reducing 4NQO with phenylhydrazine according to the method of Ochiai and Mitarashi (20).

Determination of Sulphhydryl Groups. Reactions between sulphydryl compounds and 4-substituted quinoline-1-oxides were routinely carried out for 1 hour in a water bath at 37°C. The reaction mixtures, which were similar to those adopted by Endo (3) in his study of the reaction of 4NQO and sulphydryl groups, consisted of 4 ml of a methanol solution of the appropriate amount of 4HAQO or 4AQO, 4 ml of an aqueous solution of the appropriate amount of glutathione or cysteine, 1 ml of 10⁻² M EDTA, and 1 ml of 10⁻¹ M phosphate buffer at pH 7.0. The reactivity of 4-substituted quinoline-1-oxides with sulphydryl compounds was determined by measuring unconsumed sulphydryl groups with an amperometer according to the method of Benesch et al. (2) unless otherwise indicated.

Chromatography. Paper chromatography was carried out for periods of 16 hours. Substances derived from the reaction of 4HAQO with sulphydryl compounds were detected according to the method of Okabayashi and Ide (22) using Whatman No. 1 filter paper with the upper layer of 1:1:1 mixture of ethyl acetate, sec-butanol, and water as the solvent system. Substances derived from the reaction of cysteine with 4HAQO were detected using Töyö No. 51 filter paper with a 4:1:2 mixture of n-butanol, glacial acetic acid, and water as the solvent system.

Thin-layer chromatography was carried out, on the basis of a recent report by Sugimura et al. (29) that 4HAQO and its derivatives could be separated in this way, using silica gel (Eastman

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chromatogram sheet, Type K301R2 (silica gel), without fluorescent indicator, made by Distillation Products Industries, Rochester, N. Y.) as the supporting material, with the upper layer of 1:1:1 mixture of ethyl acetate, sec-butanol, and water, or with sec-butanol saturated with water, as the developing solvent system. Times for development were 2 hours for the former system and 2.5 hours for the latter system.

RESULTS

Effects of 4HAQO and 4AQO on Sulfhydryl Compounds. Since 4NQO and some of its derivatives have been reported to react with sulfhydryl compounds at pH 7.0 at 37°C the behavior of 4HAQO and 4AQO towards sulfhydryl compounds was first observed under the same conditions. The results are summarized in Table 1. As Table 1 shows, sulfhydryl group were completely consumed by 4HAQO under the conditions adopted, but were not consumed at all by 4AQO, even when the concentration of the latter was 5 times as great as that of the sulfhydryl compounds.

Kinetics of the Oxidation of Glutathione by 4HAQO.

To determine the mathematical relationship of the rate of reaction to the relative concentration of the reactants, the initial concentration of glutathione was kept constant, and the effects of serially diluted solution of 4HAQO on the oxidation of glutathione were observed for various periods of incubation at pH 7.0 at 37°C. As Chart 1 shows, the oxidation of glutathione was not instantaneous, but was gradual, and was clearly dependent on the concentration of 4HAQO.

The rate of reaction was estimated, on the basis of Chart 1, to be almost directly proportional to the concentration of the quinoline derivative. In order to analyze the reaction more precisely, the percentage remaining unoxidized of the initial concentration of glutathione was plotted on the log scale of semilog graph paper, and the time was plotted on the linear scale. The data fall essentially on straight lines, indicating that the oxidation of glutathione by 4HAQO follows first-order kinetics. That being the case, the relationship of \( K \), the reaction constant, to \([HAQO]_0\), the initial concentration of 4HAQO, and \( t_1 \), the time required for the concentration of glutathione to decrease to half its initial value, is shown by the following equation:

\[
K = \frac{\ln 2}{[HAQO]_0 t_1}
\]

The values obtained for \( t_1 \) and \( K \) are listed in Table 2. The three values of \( K \) in Table 2 are effectively the same, showing that the oxidation of glutathione by 4HAQO is indeed a first-order reaction.

Effect of pH on the Oxidation of Glutathione by 4HAQO.

The pH of reaction mixtures was adjusted to various values between 4.5 and 7.5 either with or without phosphate buffer, and the amount of oxidation were measured for incubation at 37°C for 1 hour (Chart 2). Under the conditions adopted, the effect of pH was roughly the same in both buffered and unbuffered reaction mixtures. There was no oxidation below pH 5.0. Between pH 5.0 and 7.0, oxidation tended to increase with pH. At pH 7.0 and above, the glutathione was completely oxidized.

Detection of Products of Reaction between Sulfhydryl Compounds and 4HAQO. In order to identify all products of reaction between sulfhydryl compounds and 4HAQO, the reaction was carried out under conditions such that the sulfhydryl compounds had definitely reacted. After the reaction was completed, chromatography was carried out according to the methods of Okabayashi and Ide (22) and Sugimura et al. (29) in order to detect any possible alteration in the 4HAQO (Table 3). The \( R_f \) values obtained for reaction mixtures of 4HAQO and cysteine or glutathione were identical with those obtained for 4HAQO in the absence of any sulfhydryl compound, and with those obtained for authentic 4HAQO. They were distinctly different from those obtained for authentic 4AQO.

The absorption spectrum of 4HAQO has a maximum at 355 m\( \mu \) and a slight shoulder at 260 m\( \mu \) in neutral solution, and a

![chart](chart1.png)

**CHART 1.** Effect of concentration of 4HAQO on the oxidation of glutathione. 0.04 \( \mu \)mole/ml of glutathione was incubated with 0.04 (○—○), 0.02 (●—●), and 0.01 (▲—▲) \( \mu \)mole/ml of 4HAQO at 37°C for various times in the presence of 1 \( \mu \)mole/ml of EDTA and 10 \( \mu \)moles/ml of phosphate buffer, pH 7.0. 4HAQO, 4-hydroxyaminoquinoline-1-oxide; EDTA, tetrasodium ethylenediamine-tetraacetate; —SH, sulfhydryl compound.

<table>
<thead>
<tr>
<th>Quinoline derivatives</th>
<th>Concentration (( \mu )moles/ml)</th>
<th>Sulfhydryl compounds</th>
<th>Concentration (( \mu )moles/ml)</th>
<th>—SH oxidized (( \mu )moles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4HAQO</td>
<td>0.08</td>
<td>Glutathione</td>
<td>0.04</td>
<td>0.040</td>
</tr>
<tr>
<td>4HAQO</td>
<td>0.08</td>
<td>Cysteine</td>
<td>0.04</td>
<td>0.039</td>
</tr>
<tr>
<td>4AQO</td>
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<td>Glutathione</td>
<td>0.40</td>
<td>0</td>
</tr>
<tr>
<td>4AQO</td>
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<td>Cysteine</td>
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<td>0</td>
</tr>
<tr>
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<td>Glutathione</td>
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</tr>
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<td>Cysteine</td>
<td>0.40</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>Glutathione</td>
<td>0.04</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>Cysteine</td>
<td>0.04</td>
<td>0</td>
</tr>
</tbody>
</table>

* Abbreviations: 4HAQO, 4-hydroxyaminoquinoline-1-oxide; 4AQO, 4-aminoquinoline-1-oxide; —SH, sulfhydryl compound.
**TABLE 2**

<table>
<thead>
<tr>
<th>Concentration of 4HAQO (moles/liter)</th>
<th>$t_1$ (sec)</th>
<th>$K$ [(moles/liter)$^{-1}$ sec$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$4 \times 10^{-5}$</td>
<td>94</td>
<td>185</td>
</tr>
<tr>
<td>$2 \times 10^{-5}$</td>
<td>182</td>
<td>191</td>
</tr>
<tr>
<td>$1 \times 10^{-5}$</td>
<td>384</td>
<td>181</td>
</tr>
</tbody>
</table>

* Abbreviation: 4HAQO, 4-hydroxyaminoquinoline-1-oxide.

maximum at 345 m$\mu$ in acidic solution. These spectra were not altered by reaction with cysteine or glutathione.

Chromatography was also carried out to determine the exact fate of the cysteine after its reaction with 4HAQO. Ten ml of reaction mixture containing 0.04 μmole/ml cysteine with 0.08 μmole/ml 4HAQO in the presence of 1 μmole/ml EDTA and 10 μmoles/ml phosphate buffer, pH 7.0, were incubated at 37°C for 1 hour. After the incubation, paper chromatography was conducted at room temperature for 16 hours with an aliquot of the incubation mixture using a 4:1:2 mixture of n-butanol, glacial acetic acid, and water as the solvent system. Only one ninhydrin-positive spot was observed, and its RF value, 0.16, was identical with that of cystine. Other spots, representing products of substitution reaction between cysteine and 4HAQO comparable to the products of substitution reaction between sulfhydryl compounds and 4NO (3, 21) were not found.

**Sodium Sulfite Reduction of Sulfhydryl Compounds Oxidized by 4HAQO.** Since the sulfhydryl compounds were oxidized by 4HAQO without forming any substitution compounds and without any alteration in the 4HAQO, the role of the 4HAQO in the oxidation of the sulfhydryl compounds appeared to be purely catalytic. Oxidized sulfhydryl compounds, RSSR, are known to be reduced by sodium sulfite as follows:

$$RSSR + SO_3^- \rightarrow RS^- + RSO_3^- \quad [B]$$

This reaction was used in order to determine the nature of the role played by 4HAQO in the oxidation of sulfhydryl compounds.

Reaction mixtures consisting of 20 ml of $2 \times 10^{-4}$ M glutathione or cysteine, 5 ml of $10^{-4}$ M EDTA, and 5 ml of $10^{-4}$ M phosphate buffer at pH 7.0 were incubated routinely at 37°C for 1 hour. After incubation, 6 ml of each reaction mixture were added to a separate sample of a mixture of 0.3 ml of $10^{-4}$ M EDTA, 12 ml of ammonia buffer at pH 9.5 (2 M NH$_4$OH, 0.8 M NH$_2$NO$_3$), 12 ml of ethanol, and 3 ml of 10% Na$_2$SO$_3$.

The sulfhydryl contents of the combined reaction mixtures were determined with an amperometer. The determination was performed immediately in the case of the combined reaction mixture containing cysteine, but only after stirring for 30 minutes at room temperature in the case of the combined reaction mixture containing glutathione. The results are summarized in Table 4. According to Equation B, complete sodium sulfite reduction restores only half of the original RSSR. As Table 4 shows, both sulfhydryl compounds were oxidized completely by 4HAQO and on the basis of Equation B, the oxidation of both compounds was almost completely reversed by sodium sulfite.

**Effects of Oxygen and Light on the Oxidation of Glu-**

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**CHART 2.** Effect of pH on the oxidation of glutathione by 4HAQO. 0.04 μmole/ml of glutathione was incubated at 37°C for 1 hour with 0.04 μmole/ml of 4HAQO in the presence of 1 μmole/ml of EDTA to give the respective pH desired. O, pH adjusted with 0.1 N HCl without buffer. □, pH in the presence of 10 μmoles/ml of phosphate buffer. 4HAQO, 4-hydroxyaminoquinoline-1-oxide; EDTA, tetrasodium ethylenediaminetetraacetate; —SH, sulfhydryl compound.
The oxidation of sulfhydryl compounds by 4HAQO was found in the present study to have several interesting characteristics that are quite different from those of the reaction between sulfhydryl compounds and 4NQO (3, 21). Oxidation by 4HAQO occurred to a much greater extent in the presence of oxygen than in its absence, and did not involve any substitution reaction between the sulfhydryl compounds and the 4HAQO. After reaction, no alteration in the 4HAQO was detected, and the oxidized sulfhydryl compounds were found to be completely reduced by sodium sulfite. The kinetic data indicate that the oxidation of sulfhydryl compounds by 4HAQO is a first-order reaction, confirming the observation that no substitution reaction between the principal participants is involved, and implying that the role played by the 4HAQO is purely catalytic.

The oxidation of sulfhydryl compounds by 4HAQO was found to be markedly dependent on pH. No oxidation was detected below pH 5. Between pH 5 and 7, oxidation increased with the pH. Under the conditions adopted, sulfhydryl groups were completely consumed at pH 7. The dependence of the reaction on pH suggests that the extent to which the sulfhydryl group is dissociated may be important. Maximum dissociation of the sulfhydryl group in glutathione occurs at pH 9.2, and the closer the pH of the reaction mixture approaches this value, the faster the reaction, at least between pH 5 and 7.

On the other hand, the effect of pH may also be related to the formation of free radicals from 4HAQO. During the course of the present study, Nagata et al. (15) studied this latter reaction, which may play a role in the oxidation of biologic materials by the quinoline derivative. No indication of free radical formation was observed at pH 3, but strong indication was observed in alkaline solution.

The oxidation of sulfhydryl compounds by 4HAQO proceeded normally in the presence of oxygen, but was greatly decreased in the presence of nitrogen. Light did not significantly affect oxidation in air, but it slightly increased oxidation under nitrogen. Evidently, oxygen plays a major role in the reaction, but light plays a minor role. In air, the rate of oxidation may well be too rapid to permit detection of the effect of light.

The influence of oxygen and light on the oxidation of sulfhydryl compounds by 4HAQO suggests that the conditions under which this reaction takes place are closely related to those under which free radicals are formed from the quinoline derivative. Nagata et al. (15) observed that oxygen was necessary for the formation of free radicals from 4HAQO and that light promoted the reaction. This reaction which was also dependent on temperature, yielded free radicals (II, III) from 4HAQO (I) according to an equation in Chart 3.

Even though direct evidence has yet to be obtained, it seems reasonable to conclude, at least tentatively, that the oxidation of sulfhydryl compounds by 4HAQO proceeds through interaction of the sulfhydryl compounds with the free radical formed from the quinoline derivative. On this basis, the overall reaction between 4HAQO and sulfhydryl compounds may be shown in Chart 4. The difference between 4HAQO and 4NQO with regard to their modes of reaction with sulfhydryl compounds is worthy of attention in connection with their different potencies in carcinogenesis. According to Endo and Kume (6) and Shirasu (27), the carcinogenic potency of 4HAQO is definitely greater than that of 4NQO, and this difference between the carcinogenic poten-
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