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ENZYME-INDUCED AZIRIDINE FORMATION BY RAT LIVER MICROSOMES

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The metabolic reaction of 2-chloroethylaminobenzoquinone derivatives with rat liver microsomes was studied. In buffer solution containing cellular macromolecules such as DNA or protein, 2-chloroethylaminobenzoquinones were unusually stable and could be recovered quantitatively after an appropriate period of incubation. However, in microsomal solution, they were gradually converted into aziridinobenzoquinones via elimination of hydrogen chloride by NADPH-dependent enzyme action. Based on the study of the enzymatic cyclization reaction of 4-chlorobutylaminonaphthoquinone, we concluded that the hydroquinone derivative is an important intermediate for aziridine formation.

2,5-Bis(2-chloroethylamino)-p-benzoquinone 1 or 2-chloroethylamino-naphthoquinone 2 has been known as one of the most highly bioactive compounds for more than 20 years. Even a very dilute solution of less than 10^{-6} molar of 1 is enough to inhibit growth of Yoshida sarcoma of rat *in vitro* (1).

The antitumor activity of chloroethylaminoquinone derivatives including 1 or 2 are generally nearly comparable to the value observed for their aziridine derivatives such as 5 or 6. Consequently, the chloroethylamino group may be converted into the aziridine ring at the first step of their metabolic reaction although we have found no report of a study of this. Strangely enough, however, 1 or 2 is unusually stable chemically in the biomimetic condition and is quantitatively recovered from the incubation mixture if there is no enzymatic action. Recently, we also observed that the chlorine atom of 2 is completely inert to the exchange reaction with Cl^{36} in buffer solution (2). This should eliminate even the possibility of

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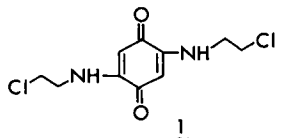
the formation of aziridinium salt 3 in the cellular system and coincides well with the above.



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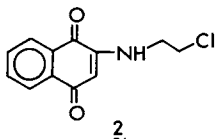

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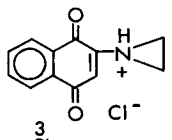

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We confronted the question of why compound 1 or 2 is bioactive and report here on the enzyme-induced aziridine formation from 2-chloroethylaminobenzoquinones or naphthoquinones.

MATERIALS AND METHODS

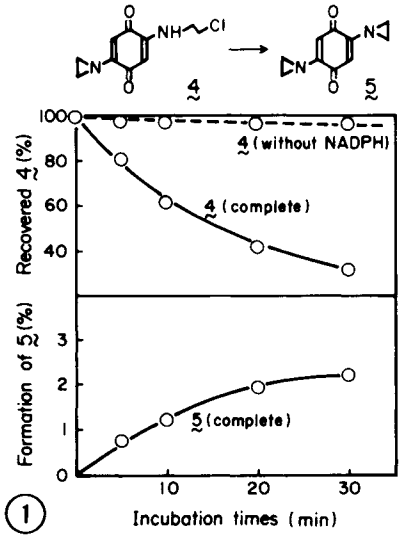
2,5-Bis(2-chloroethylamino)-p-benzoquinone (1), 2,5-bis(1-aziridinyl)-p-benzoquinone (5), 2-chloroethylaminonaphthoquinone (2), and 1-aziridinylnaphthoquinone (6) were prepared by the method of Petersen et al. (1). 2-(2-Chloroethylamino)-5-aziridinyl-p-benzoquinone (4) and 2-bromoethylaminonaphthoquinone (7) were prepared from the corresponding aziridines 5 and 6 with concentrated HX acid in acetonitrile. 4, m.p. 115-116°C, orange plates ($CHCl_3$); 7, m.p. 182-3°C, red plates (EtOH). 4-Chlorobutylaminonaphthoquinone (8) was prepared by addition reaction of 4-aminobutanol to naphthoquinone followed by chlorination with thionylchloride in dichloroethane. m.p. 139-140°C, red plates (MeOH). Pyrrolidinylnaphthoquinone (9) was prepared by addition reaction of pyrrolidine on naphthoquinone in ethanol (1). m.p. 158.5-159.5°C, red plates (MeOH). Authentic sample of 12 (n = 4) was prepared by treating 9 with $NaBH_4$ followed by the dimethylsulfate reaction. Colorless needles, m.p. 85-86°C for 12 (n = 4) (MeOH).

General procedure of transformation of chloroethylamino- or aziridinyl-p-benzoquinones or naphthoquinones in microsomal suspension. Washed liver microsomes were prepared from normal SLC-Wistar strain male rats, weighing about 300 g, by the differential centrifugation method (3). The protein concentration of the microsomes was determined by the biuret reaction (3) using bovine serum albumin as a standard.

The metabolic reactions were carried out in a complete system consisting of 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl, 10 mM $MgCl_2$, 1 mM nicotinamide, 1 mM pyrophosphate, 30 mg of protein of microsomes, 0.0026 mM of substrates and an NADPH-generating system (0.9 mM NADP, 10 mM glucose-6-phosphate and 12.5 units of glucose-6-phosphate dehydrogenase) in a final volume of 5 ml. Unless otherwise stated, incubation was carried out aerobically at 37°C with moderate shaking. This procedure was almost the same as that described previously in the study of the aziridine derivative (4). To stop the enzymatic reaction, aliquots portion of the reaction mixture were added to twice the volume of ice-cooled acetonitrile. The top clear solution obtained by centrifugation was analyzed by HPLC using Nucleosil$_{10}$ C_{18} column. The reaction products were identified mainly by GC/MS or comparison of the physical data with those of authentic samples. The yields of the reaction products were calculated from the HPLC peak areas.

RESULTS

A) Reaction of 2-(2-chloroethylamino)-5-aziridinyl-p-benzoquinone 4 and 2,5-bis(1-aziridinyl)-p-benzoquinone 5 in the complete, NADPH-free or



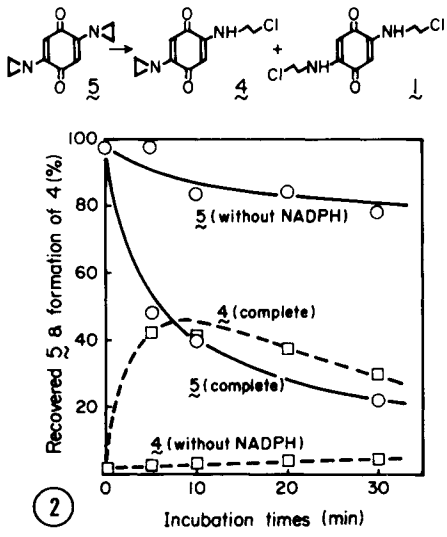


Fig. 1. Reaction of 4 in the complete or NADPH free system (37°C)

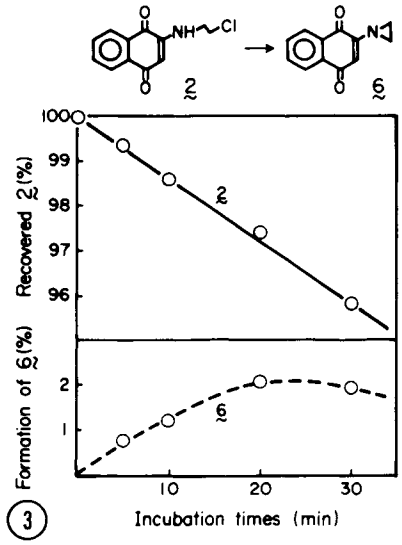


Fig. 3. Reaction of 2 in the complete system (37°C)

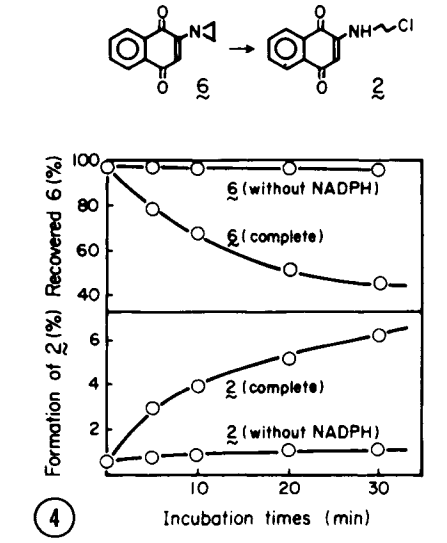


Fig. 2. Reaction of 1 in the complete or NADPH free system (37°C)

Fig. 4. Reaction of 6 in the complete or NADPH free system (37°C)

microsomes free system (Figs. 1 and 2). At first, 4 was treated with rat liver microsomes in the complete system. The reaction products were 2,5-bis(1-aziridinyl)-p-benzoquinone 5 and a small amount of 2,5-bis(2-chloroethylamino)-p-benzoquinone 1. The amounts of recovered 4 and the yield of 5 from the reaction mixture incubated for appropriate periods are shown in Fig. 1. The NADPH-free or microsome-free system showed no reaction on 4 nor

formation of 5. The conversion of 4 into 5 therefore clearly proceeded by the enzymatic process.

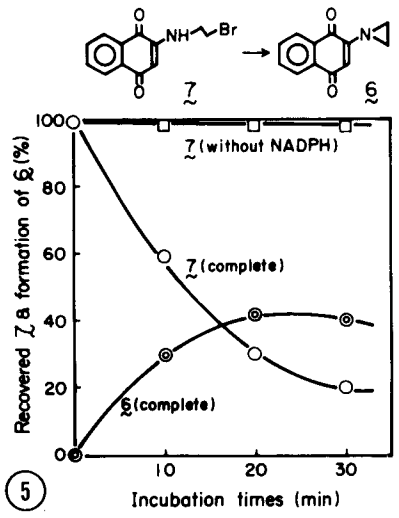
Consumption of 5 occurred under nonenzymatic conditions even though it was very slow and accelerated strongly by the addition of NADPH. The main product was 4 which may be formed by the reaction with chlorine ion of the buffer solution.

B) Reaction of 2-chloroethylaminonaphthoquinone 2 and 2-aziridinyl-naphthoquinone 6 in the complete or nonenzymatic system (Figs. 3 and 4). The reaction of naphthoquinone derivatives 2 and 6 in the complete system was essentially similar to that of the benzoquinone derivatives. The formation of 6 from 2 was only observed in enzyme-active solution and consumption of 6 or formation of 2 was also accelerated by the addition of NADPH.

C) Reaction of 2-bromoethylaminonaphthoquinone 7 in the complete system (Fig. 5). We expected better yield of aziridine than chloro derivatives by cyclization of the bromoethylamino group. Our result coincided well the expectation and 7 gave a good yield of aziridine 6. Compound 7 was completely inert in the NADPH-free or microsome-free system.

D) Cyclization reaction of 4-chlorobutylaminonaphthoquinone 8 in the complete system (Fig. 6). Formation of a five-membered ring is usually much easier than that of aziridines. This tendency was also observed in the enzymatic cyclization of 8, which disappeared completely during 20 min of incubation at 37°C and gave 60% of cyclization product 9. Probably oxidative consumption of 8 and 9 decreased the yield of 9. In NADPH free or microsome-free system, 8 was stable and was recovered quantitatively after a prolonged period of incubation.

E) Reaction of 2-chloroethylaminonaphthoquinone 2 by deactivated microsomes (Fig. 7). Enzymatic activity of microsomes to produce the aziridine ring system from 2-chloroethylaminoquinones was rapidly lost upon contact of the microsomes with air at 37°C. Thus, delayed addition of substrate into the complete system under aerobic condition strongly depressed the formation of product 6. At 60 min after contact with air, microsome



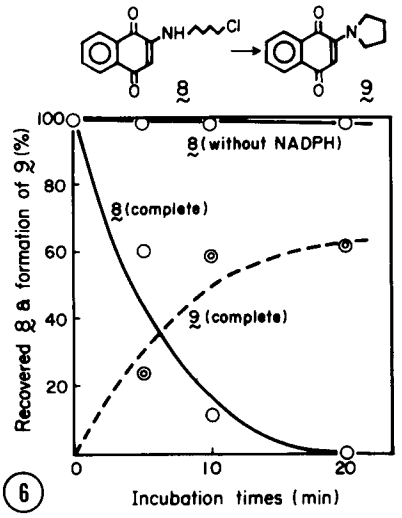


Fig. 5. Reaction of 7 in the complete or NADPH free system (37°C)

Fig. 6. Reaction of 8 in the complete or NADPH free system (37°C)

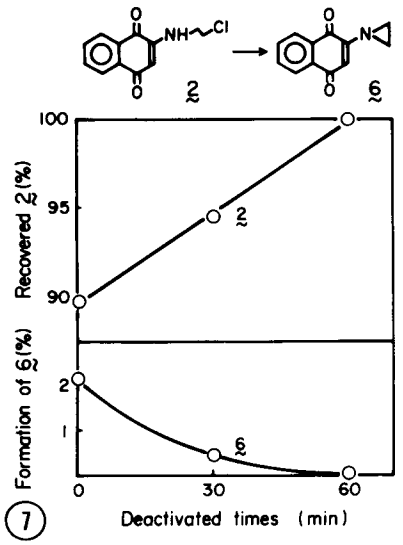


Fig. 7. Reaction of 2 by a deactivated microsomes in the complete system for 20min at 37°C

activity completely disappeared and starting material was recovered quantitatively.

F) Enzymatic cyclization in different gas phases. Aziridine formation was slightly accelerated by changing the gas phase from air to argon or carbon monoxide. The amount of recovered starting materials also increased,

$\underset{\sim}{2} \longrightarrow \underset{\sim}{6}$

Gas Phase	Recovered 2(%)	Yield of 6(%)
Air	91.3	2.4
Argon	96.5	2.9
CO	96.8	3.1

$\underset{\sim}{4} \longrightarrow \underset{\sim}{5}$

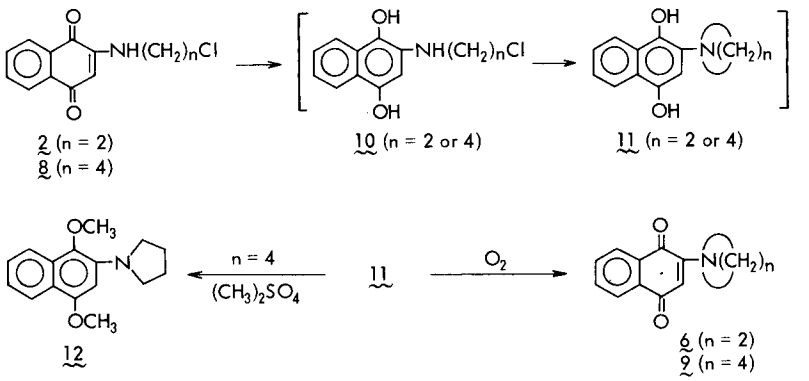
Gas Phase	Recovered 4(%)	Yield of 5(%)
Air	40.3	2.0
Argon	43.4	3.4
CO	44.2	4.5

Table I. Reaction of $\underset{\sim}{2}$ & $\underset{\sim}{4}$ by the complete system under different gas phases (20min, 37°C)

as shown in Table 1. Aziridine formation by a reductive enzymatic process and oxidative consumption of the starting materials and the products were clearly indicated.

G) $NaBH_4$ reduction of naphthoquinone derivatives and detection of hydroquinones as an intermediate of enzyme-induced aziridine formation (5). In studying the reaction of $\underset{\sim}{2}$ or $\underset{\sim}{8}$ with microsomes, we have usually observed decolorization of the characteristic color of quinone derivatives. This suggested that the enzyme-induced cyclization reaction proceeds via formation of leuco compound due to reduction of the quinone ring by quinone reductase of microsomes. Thus, we tried the reaction of $\underset{\sim}{2}$ and $\underset{\sim}{8}$ with $NaBH_4$ in aqueous buffer solution. After addition of $\underset{\sim}{2}$ or $\underset{\sim}{8}$ to $NaBH_4$ solution, as expected, the reddish color of these compounds disappeared immediately and then colored products $\underset{\sim}{6}$ or $\underset{\sim}{9}$ formed upon contact of the reaction mixture with oxygen in the air. The structure of the leuco intermediates was estimated to be aziridinyl or pyrrolidinyl-1,4-dihydroxynaphthalene $\underset{\sim\sim}{11}$ (n = 2 or 4) by studying their methylation products $\underset{\sim\sim}{12}$ (n = 4) by comparison with authentic samples. After this, $\underset{\sim\sim}{12}$ (n = 4) was also identified in the

enzymatic reaction solution of 8 using the complete system according to the methylation method described by Brochmann-Hanssen and Oke (6). Consequently, we concluded that 2-chloroethylamino- or 4-chlorobutylamino-naphthoquinone 2 or 8 was basically very stable and no reaction occurred. However, when naphthoquinone moiety was reduced to dihydroxynaphthalene by $NaBH_4$ or by reductase of microsomes (7), part of it may be converted into aziridinyl- or pyrrolidinyl derivative 11 (n = 2 or 4) by elimination of



hydrogen chloride. We supposed that the cyclization reaction was caused by increasing the basicity of the nitrogen atom adjacent to the reductive quinone ring (8).

CONCLUSION AND DISCUSSION

The chloroethylamino group on benzoquinone or naphthoquinone is unusually stable and inert to cellular macromolecules in buffer solution. Nevertheless, 1, 2 and 7 are strongly cytotoxic to cultivated cells such as Hela or L-1210. Study of the metabolic reaction of 2, 4 or analogous compounds solved this contradiction. Reduction of the quinone moiety into hydroquinone by enzymatic action strongly accelerated the successive cyclization reaction to form aziridine or pyrrolidine in these compounds. Furthermore, we observed that aziridines are consumed successively by other oxidative enzymatic process.

We previously proposed that the cytotoxicity and probably carcinogenicity, too, of aziridine derivatives are mainly caused by the production of nitroso compound by oxidative fragmentation of the aziridine ring (4,9).

Our results in this study coincide well with our hypothesis (9). The possibility of the formation of aziridine derivatives from inert compounds by enzymatic action is of interest in the designing of new antitumor agents.

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2. Compound 2 in the complete system containing 6.24 μ Ci of Cl_{36} as HCl was incubated for 30 min at 37°C. The yield of 6 was 6% and recovered starting material 2 was 77%. The amount of radioactive chlorine atom in recovered 6 did not exceed the natural abundance value.
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7. As an intermediate, semiquinone was proposed by Tomasz, M., et al. (1974) Biochemistry 13, 4878-4887. In our system, the presence of the semiquinone radical was not proven by direct esr detection. Our results suggested hydroquinones as intermediates.
8. This assumption was supported by CNDO/2 calculation of 2-amino-1,4 dihydroxynaphthalene and 2-aminonaphthoquinone. Yamakawa, M. (Shionogi Res. Lab.) unpublished.
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