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ENZYME-INDUCED AZIRIDINE FORMATION BY ISOLATED HEPATOCYTES

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The metabolism of 2-bromoethylaminonaphthoquinone in hepatocytes isolated from rats was studied. This compound was chemically inert in the reaction system used. However, in buffer solution containing isolated hepatocytes, it was gradually converted into aziridinylnaphthoquinone. Under the same reaction conditions, 4-chlorobutylaminonaphthoquinone also gave the cyclization products, pyrrolidinylnaphthoquinone. Cellular GSH decreased in both reactions.

Studies on the in vivo formation of aziridine derivatives which are strong antitumor agents, are very interest in research on the development of antitumor drags. We report here aziridine formation using hepatocytes isolated from rats. To our knowledge, this is the first time that aziridine formation has been observed in living cells.

Materials and Methods

2-Bromo- 1 and 2-chloroethylaminonaphthoquinone, 1-aziridinylnaphthoquinone 2, 4-chlorobutylaminonaphthoquinone 3 and 1-pyrrolidinylnaphthoquinone 4 were prepared as described previously.[1] Bovine serum albumin, 3-methylcholanthrene and sodium phenobarbital were commercially available.

Hepatocytes were obtained from male Wistar rats (300-330 g), fed ad lib, by collagenase perfusion according to Moldéus et al.[2] The cells were suspended in Krebs-Henseleit buffer, pH 7.4, containing 1% bovine serum albumin, 10 mM glucose, 13 mM Hepes and amino acid mixture (Gibco) and were incubated at 37°C in a rotating round-bottom flask at 3 x 10^6 cells/ml.[3] The viability of the cells was monitored periodically by NADH penetration test.[4]

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After the incubation period, an aliquot was quenched with the same quantity of cold acetone and the supernatant fluid, obtained by centrifugation for 5 min at 2000 rpm at 4°C, was analyzed by HPLC using Nucleosil $_{10}$C18, 50% CH_3CN-H_2O 1 ml/min by observation of the absorbance at 254 nm for quinone derivatives. GSH and GSSG analyses were carried out according to Saville,s method[5] and O-phthalaldehyde method respectively.[6] The values of the initial concentration of substrates, products and GSH shown in the figures were obtained from the solution quenched by acetone immediately after addition of substrate to the reaction system at 37°C. The values for 1 or 3 corresponded to about 98% of the theoretical concentration expected from using substrates.

Results

Metabolism of 2-bromoethylaminonaphthoquinone 1 in isolated hepatocytes. Bromoethylaminonaphthoquinone 1 was consumed smoothly under incubation at 37°C as shown in Fig. 1. The initial concentration of 481 nmole/ml for 1 decreased to 334 nmole/ml (70%) after 30 min of incubation. Accompanying this, 31 nmole/ml of aziridinylnaphthoquinone 2 was formed. The yield of 2 was 6.3% on calculation from the starting

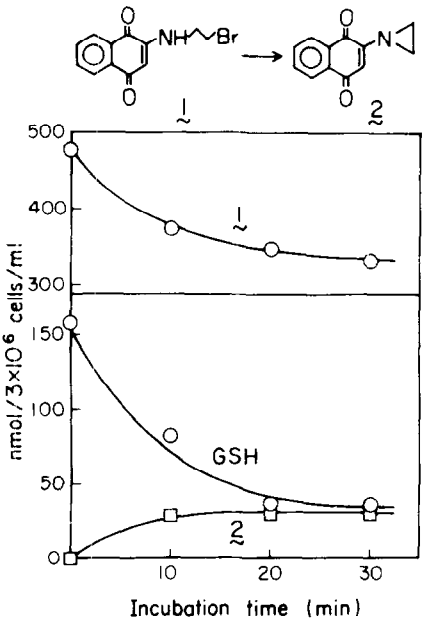


Fig 1. Metabolism of 1 in isolated hepatocytes (37°C). (The results of experiment typical of six are shown)

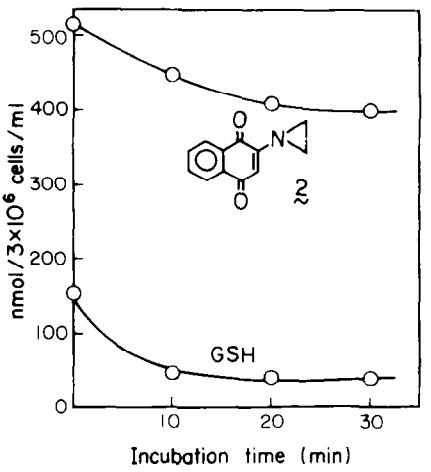


Fig 2. Decrease of 2 & GSH during incubation in isolated hepatocytes (The results of one experiment typical of five are shown)

amount of substrate 1.[7] GSH, initially present at 168 nmole/ml, also decreased to 35.6 nmole/ml at 30 min of incubation. We observed no formation of GSSG by our analysis method using HPLC. If GSSG had been formed during our metabolic reaction, it should not have exceeded 1% against GSH, the maximum limiting value allowed by our analysis method.

Cell viability was kept at about the starting value throughout the reactions.[8] Exception was only single case among six separate experiments in which cell viability was 60% at 30 min incubation although the value at 16 min was 97%. The decrease could be attributed to the formation of aziridine 2, a strong cytotoxic compound.

As shown in Fig. 2, 2 was slowly consumed when added to hepatocytes, accompanied by the decline of GSH. The viability of the hepatocytes and the formation of GSSG were also essentially similar to the reaction of 1. In a separate experiments, the hepatocytes has been isolated from phenobarbital-treated rats and 3 MC-treated rats. The yields of aziridinylnaphthoquinone in these experiments did not differ greatly from the results obtained with hepatocytes isolated from normal rats.

Metabolism of 2-chlorobutylaminonaphthoquinone 3 in hepatocytes isolated from normal rats.

The reaction of 3 in hepatocytes was carried out under the reaction conditions described for compound 1 giving the results shown in Fig. 3 and 4. The yield of the cyclization product, pyrrolidinylnaphthoquinone 4, was generally higher in several experiments than that of the aziridine ring formation from 1.[7] Better stoichiometric results due to the product yield and high recovery percentage of the starting material indicated that 3 and 4 were inert to other successive

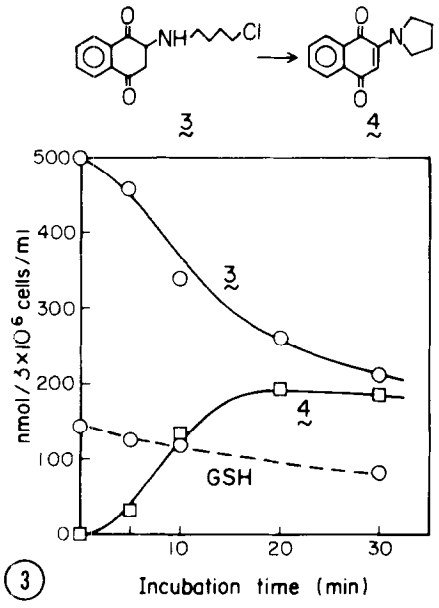


Fig 3. Metabolism of 3 in isolated hepatocytes (37°C).(The results of one experiment typical of six are shown)

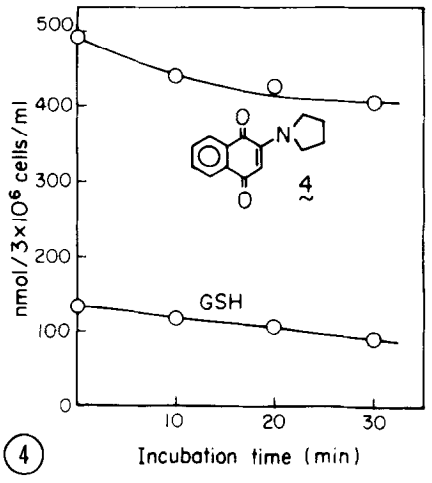


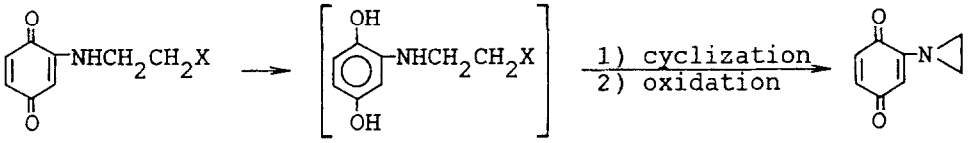
Fig 4. Decrease of 4 & GSH during incubation in isolated hepatocytes (The results of one experiment typical of five are shown)

reactions. The small decline percentage of GSH also agreed well with the low cytotoxicity of pyrrolidinylnaphthoquinone 4.[9] Viability and GSSG were essentially the same as that for the reaction of 1.

Discussion

2-Bromoethylamino- and 4-chlorobutylaminonaphthoquinone 1 and 3 were completely inert chemically under the conditions used in our metabolic reaction. When hepatocytes had been heated for 2 min at 100° prior to the metabolism study, substrate was recovered in good yield without any cyclization product after prolonged incubation. In a separate experiment, 2-chloroethylaminonaphthoquinone mixed with hepatocytes produced no aziridine. This coincided to the tendency for microsomes to usually give very poor yields of aziridine 2. These findings were very similar to those of the metabolic reaction

carried out in a microsomal solution reported previously.[1] Thus, in the initial stage of the reaction, the benzoquinone ring of these substrates probably was reduced to hydroquinone by quinone reductase of hepatocytes and consequently the reactivity of nitrogen of the haloethylamino group was enhanced to accomplish the cyclization to form aziridines.



Study of the cytotoxicity of 1 and its analogue indicated very weak activity on cultivated cells such as Hela and L-1210.[9] This can be expected as their chemical reactivities are low. However, their cyclization products, aziridinylnaphthoquinone 2 and its analogue, are very active biochemically and many derivatives have been used extensively as commercial antitumor agents. Our results on enzyme-induced aziridine formation may be helpful in the designing of new antitumor agents. Further studies are required to elucidate the structures of the binding compound for 2 and GSH.

References

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5. Saville, B. (1958) Analyst 83, 670-672.
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7. The yields of aziridinylnaphthoquinone 2 and pyrrolidinylnaphthoquinone 4 varied, giving randam values between 1-6% for 2 and 2-40% for 4. They depended on the rats used in the experiments but in general the yield of 4 tended to be better than that of 2.
8. Cell viability was usually better than 97%.
9. Hata, Y., Watanabe, M. Shiratori, O., Takase, S., unpublished data.