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Metabolism and Cytotoxicity of Hydrazine in Isolated Rat Hepatocytes¹⁾

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The relationship between the metabolism and hepatotoxicity of hydrazine (Hz) was studied in an isolated rat hepatocyte system. After incubation at 37°C for 60 min, the concentration of Hz decreased to 34% of the initial value (from 98 to 33 nmol/ml). A further pronounced loss was observed when Hz was incubated with hepatocytes isolated from rats which had been pretreated with phenobarbital (PB) or rifampicin. Since metyrapone and piperonylbutoxide inhibited the metabolism in the systems obtained from both PB-pretreated rats and untreated rats, an important role of hepatic cytochrome P-450 in Hz oxidation was suggested. The difference spectrum of Hz with cytochrome P-450 in isolated rat hepatocytes demonstrated the formation of complexes between metabolic intermediates of Hz and cytochrome P-450. The trypan blue exclusion test, the measurement of K^+ level and the determination of glutathione content retained in the cells were also performed. The results suggested that Hz is cytotoxic to isolated rat hepatocytes.

Keywords—hydrazine; metabolism; cytotoxicity; hepatocytes; rat

Hydrazine (Hz) is a toxic and hazardous metabolite of isoniazid (INH) causing fatty liver and liver necrosis, and it is also a mutagen and a carcinogen.^{2,3)} It has been detected by gas chromatography-mass spectrometry (GC-MS) in the urine of INH-dosed patients with tuberculosis.⁴⁾ It is also well-known that liver damage is frequently induced in patients on INH treatment. Thus, the toxicity of Hz and its derivatives is of great interest from the point of view of the side effects in INH therapy. Although the exact sequence of events leading ultimately to liver injury has not been fully elucidated, Timbrell *et al.* postulated that INH-induced liver necrosis may be caused by a chemically reactive metabolite of INH, monoacetylhydrazine (AcHz).⁵⁾ On the other hand, we found that Hz induced more marked hepatic injury than AcHz, and analogous but more extensive necrosis took place after Hz administration to rabbits pretreated with an inducer of cytochrome P-450 [phenobarbital (PB) or rifampicin (RMP)].⁶⁾ Liver function tests and histological studies indicated similar liver injury in rats to that in rabbits.⁷⁾ We also found that the liver and plasma concentrations of Hz in both animals after the intraperitoneal injection of INH were almost the same as those of AcHz.⁸⁾ In the pretreated rats, the liver and plasma Hz concentrations were considerably lower than those of the control group, but this was not the case with AcHz.

These facts prompted us to compare the cytotoxicities of Hz and AcHz using an isolated rat hepatocyte system. The previous experiments showed that the incubation of Hz and AcHz in isolated rat hepatocytes induced marked concentration-dependent cell death, as indicated by a trypan blue exclusion test.⁹⁾ This test did not show a distinct difference between Hz and AcHz in terms of the extent of cell injury. However, our preliminary investigations have also

shown that a significant time- and concentration-dependent depletion of hepatocellular reduced glutathione (GSH) levels was induced by Hz treatment, while AcHz caused very little GSH depletion.⁹⁾

The data suggest that a key step of Hz metabolism produces the toxic intermediate(s). As the ultimate metabolite of Hz is known to be nitrogen (N_2),¹⁰⁾ the induction of hepatotoxicity by Hz may be due to a metabolic intermediate formed during the microsomal oxidation process. Recently, we reported that Hz metabolism in rat liver microsomal fractions resulted in the formation of Hz radical and diimide.^{11,12)} Therefore, in order to clarify the relationship of Hz metabolism to its hepatotoxicity, the present examination was designed to explore the toxic effects of Hz on isolated rat cells.

Materials and Methods

Chemicals—All chemicals were commercial products of reagent grade.

Animal Treatment—Male Wistar rats weighing 250–300 g were used. As for PB-pretreated animals, sodium PB was given intraperitoneally (i.p.) at a daily dose of 50 mg/kg for 3 d. In the case of RMP-pretreated animals, 30 mg/kg of RMP was given i.p. in 5 ml of HCl solution (pH 3.0). The control group received the vehicle only.

Preparation and Incubation of Isolated Rat Hepatocytes—The hepatocytes were isolated by the collagenase perfusion method according to the previously reported protocols.⁹⁾ The yield of each preparation was $2\text{--}4 \times 10^8$ cells/liver. Viability of the cells was determined by means of the trypan blue exclusion test.¹³⁾ Hepatocytes with a viability of more than 95% were used for the experiments. The isolated cells were suspended in Krebs–Henseleit buffer (pH 7.4) containing 1% bovine serum albumin, 10 mM glucose and 13 mM Hepes. Then 4×10^6 cells/ml were incubated at 37 °C in rotating round-bottomed flasks under an atmosphere of 95% O_2 –5% CO_2 . Prior to the addition of the test compounds, the cells were allowed to equilibrate for 3 min at 37 °C.

Hz Metabolism in the Hepatocyte System—Hz sulfate dissolved in the incubation medium was added to the hepatocyte system to a final concentration of 0.1 mM. After incubation for 15, 30, and 60 min, 1 ml of 40% zinc sulfate and 2 ml of saturated barium hydroxide were added to 1 ml of the incubation mixture with ^{15}N -Hz (1 μ g/ml) as an internal standard. The resultant precipitate was sedimented by centrifugation, and an aliquot of the supernatant fluid was extracted twice with 20 ml of ethyl acetate. The combined extract was evaporated to dryness under a stream of nitrogen. Determination of Hz was performed by GC-MS as already reported.¹¹⁾

Evaluation of Toxicity to Hepatocytes—The incubation at 37 °C was started within 1 h after the final resuspension of the cell pellets. Hz or AcHz was added to the rotating round-bottomed flasks to make a final concentration of 1 mM, and the suspension was incubated for 1 h under an atmosphere of 95% O_2 –5% CO_2 . The solution was sampled at 0, 15, 30 and 60 min and cell damage was checked by means of the trypan blue exclusion test.¹³⁾ The cells were separated from the reaction medium and the intracellular K^+ was estimated by the method described by Baur *et al.*¹⁴⁾ A 400 μ l polyethylene microcentrifuge tube was filled with 100 μ l of 0.7 M perchloric acid (PCA) solution followed by 200 μ l of 1-bromododecane on top of it. On top of the oil was placed a 100 μ l aliquot of cell suspension. Centrifugation was carried out at 10000 rpm for 10–15 s. The K^+ in the PCA layer (cell portion) was analyzed by flame photometry (Shimadzu AA-640-12 flame photometer). GSH and oxidized glutathione (GSSG) analyses were carried out according to Watanabe *et al.*¹⁵⁾

Measurements of Rat Hepatic Cytochrome P-450 Contents and the Difference Spectrum of Hz with the Cytochrome P-450—The total amount of cytochrome P-450 was assayed by using hepatocytes disrupted by freezing and thawing three times, by the method described in the previous paper.⁶⁾ The difference spectrum with cytochrome P-450 was measured with a Shimadzu MPS 2000 spectrometer.¹¹⁾ The experiment was performed in the hepatocyte system described in the legend to Fig. 3.

Results and Discussion

Figure 1 shows the time course of Hz disappearance after the incubation of 0.1 mM Hz with hepatocytes isolated from intact, PB- or RMP-pretreated rats. After incubation for 60 min with hepatocytes from the untreated group, the Hz concentration had decreased to 34% of the initial value (from 98 to 33 nmol/ml), while the Hz levels after 60 min with hepatocytes from PB- and RMP-pretreated rats were 21% and 29% of the initial value, respectively (significantly different from the untreated group). The half-lives of Hz disappearance were calculated as 0.71 h for the control group, 0.47 h for the PB-pretreated group

and 0.60 h for the RMP-pretreated one. The significant decrease in the latter two cases can be reasonably explained in terms of the increase of the hepatic cytochrome P-450,¹⁶⁾ since the main metabolic pathway of Hz is cytochrome P-450 dependent oxidation.¹⁰⁾

In the previous study *in vivo*, we observed that the elimination rate of Hz was significantly increased in PB- and RMP-pretreated rats after intravenous administration.⁸⁾ In order to clarify the metabolic course of Hz, the effects of metyrapone and piperonylbutoxide, potent inhibitors of cytochrome P-450, were investigated using isolated rat liver cells. As shown in Fig. 2A, the decrease of Hz in the hepatocytes obtained from the control group was retarded by piperonylbutoxide addition (0.1 mM), while metyrapone (1.0 mM) did not have a significant effect. The difference between the inhibitory effects of these treatments may result from a difference in the extents of hepatocyte uptake of the respective compounds or inhibition of a specific isozyme of cytochrome P-450 which participates in the Hz oxidation. Figure 2B shows the effect of metyrapone on Hz disappearance in hepatocytes isolated from PB-pretreated rats

Fig. 1. Time Course of Hz Disappearance in Isolated Rat Hepatocytes

●, normal hepatocytes; ▲, PB-pretreated hepatocytes; ■, RMP-pretreated hepatocytes. The initial concentration of Hz was 0.1 mM. Each value represents the mean of 6–10 experiments. a) $p < 0.05$; b) $p < 0.01$ (significant difference from the percentage of Hz in the normal hepatocytes).

Fig. 2. Effects of Metabolic Inhibitors on Hz Disappearance in Isolated Rat Hepatocytes

A: normal hepatocytes. B: PB-pretreated hepatocytes. ●, Hz; ○, Hz + metyrapone (1 mM); ■, Hz + piperonyl butoxide (0.1 mM). The initial concentration of Hz was 0.1 mM. Each value represents the mean $\pm$ S.E. of 5–10 experiments. a) $p < 0.05$; b) $p < 0.01$ (significant difference from the percentage of Hz without metabolic inhibitors).

in which the content of hepatic cytochrome P-450 ($1.45 \text{ nmol}/10^6 \text{ cells}$) was twice that of the control rats ($0.72 \text{ nmol}/10^6 \text{ cells}$). The Hz disappearance rate was significantly decreased by metyrapone addition in the hepatocytes isolated from PB-pretreated rats. Therefore, the results suggest an important role of PB-inducible cytochrome P-450 in Hz oxidation.

Therefore, we examined the difference spectrum of Hz with cytochrome P-450 in the hepatocytes. After immediate appearance of the type-II spectrum of Hz and the oxidized cytochrome P-450 (Fig. 3A), the formation of complexes between the metabolic intermediates of Hz and cytochrome P-450 was observed as a shift of the strong absorption maximum at 446 to 444 nm, which changed time-dependently from state B to state C in 8 min and thence to state E as shown in Fig. 3. Using rat liver microsomes, we previously demonstrated the formation of diimide ($\text{HN}=\text{NH}$) as one of the metabolites of Hz.¹¹⁾ Diimide affords an unstable complex with cytochrome P-450, showing an absorption maximum at 448 nm during the first 5 min. The peak at 448 nm was significantly inhibited by metyrapone. The observation of the peak at 446 nm in the present measurement supports the formation of diimide in the hepatocyte system. On the other hand, the peak at 444 nm given by the more stable complex (D and E) was not observed in the microsomal examination. Therefore, this complex is probably formed through a reaction catalyzed by an enzyme which is not present in the microsomal system but is in the hepatocytes. Furthermore, Hz radical ($\text{H}_2\text{N}-\dot{\text{N}}\text{H}$) has also been detected in rat liver microsomes as a reactive intermediate after incubation.¹¹⁾ Later it was found that the same radical could also be formed by one electron oxidation catalyzed only by reduced nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P-450 reductase (fp_2) without cytochrome P-450 addition.¹⁷⁾ The significance of this is now under investigation.

In order to clarify the relationship between metabolism and cytotoxicity of Hz in the hepatocyte system, we carried out toxicological evaluation tests such as the trypan blue exclusion test and measurements of intracellular K^+ ion and glutathione levels. Figure 4 indicates the cell viability and the amount of K^+ retained in the hepatocytes isolated from PB- and RMP-pretreated rats after incubation with Hz at 37°C for 15, 30 and 60 min. PB

Fig. 3. Interaction of Hz with Cytochrome P-450 in Hepatocytes Isolated from PB-Pretreated Rats

A 4.8 ml aliquot of the suspension ($3 \times 10^6 \text{ cells/ml}$) was divided into two cuvettes. After recording a flat base-line, 0.1 ml of Hz solution was added at a concentration of 1.0 mM to the sample cuvette and an equal volume of buffer solution was added to the reference cuvette. The difference spectra were recorded at 37°C just after shaking the sample solution.

A, 2 min; B, 5 min; C, 8 min; D, 15 min; E, 20 min.

Fig. 4. Hz Toxicity Measured in Terms of Trypan Blue Viability Test (A) and Percentage of Intracellular K⁺ (B)

A concentration of 1.0 mM Hz was employed. Each bar represents the mean percentage $\pm$ S.E. ($n=3-4$) with respect to the 0 time value at successive time points (15, 30 and 60 min) during the experiments. *a*) $p < 0.05$ (significant difference from the value in the normal hepatocytes at each time point).

Fig. 5. Concentration Dependence of Toxicity of Hz (□) and Acetyl Hz (▨) Measured in Terms of the Trypan Blue Viability Test (A) and Percentage of Intracellular K⁺ (B) in the Normal Hepatocytes after Incubation at 37 °C for 30 min

Each bar represents the mean percentage $\pm$ S.E. ($n=4-5$) with respect to the 0 time value without incubation in each hepatocyte system. The control experiment was performed by the addition of the incubation medium instead of the substrate solution.

pretreatment had a marked effect on the toxicity, while RMP pretreatment had only a slight effect. Therefore, the concentration dependence of the toxic effects of Hz was examined and compared with that of AcHz. Since the results showed that the cell viability and the amount of K⁺ were reduced concentration-dependently by both Hz's (Fig. 5), cytotoxicity was demonstrated. However, no difference of cytotoxicity could be seen between Hz and AcHz.

The intracellular GSH content was significantly decreased not only with increasing Hz concentrations in the mixture but also with increasing incubation time with the hepatocytes isolated from both PB- and RMP-pretreated rats (Fig. 6). We observed no significant increase in the formation of GSSG, as shown in Table I. A similar tendency was reported by Watanabe

Fig. 6. Effect of Hz on GSH in Hepatocytes Isolated from PB- and RMP-Pretreated Rats

A concentration of 1.0 mM Hz was employed. Each bar represents the mean percentage $\pm$ S.E. ($n=5-6$) to the 0 time value without incubation at successive time points (15, 30 and 60 min) during incubation at 37 °C. *a)* $p < 0.05$ (significant difference from the value obtained from the normal hepatocytes at each time point).

TABLE I. GSH and GSSG Levels in Isolated Rat Hepatocytes

	Glutathione (nmol/ 10^6 cells)		
	Total	Reduced	Oxidized
Control	123.92 ± 1.74	64.04 ± 4.52	26.89 ± 6.55
Hz (1 mM)	$92.00 \pm 10.58^{a)}$	$46.69 \pm 4.38^{b)}$	28.73 ± 4.30

Each value represents the mean $\pm$ S.E. of six experiments. *a)* $p < 0.01$; *b)* $p < 0.05$ (significant difference from the control value obtained from normal hepatocytes).

et al. in connection with the metabolism of 2-bromoethylaminonaphthoquinone in isolated rat hepatocytes.¹⁵⁾ Interestingly, AcHz did not have any effect on GSH content, as has already been reported by us.⁹⁾ The reason for the discrepancy in the effects of Hz and AcHz on GSH content remains to be clarified. In any case, the complex formation between the metabolic intermediate of Hz and cytochrome P-450 and the subsequent depletion of GSH may reflect Hz-induced hepatotoxicity, as reported in the case of SKF 525-A.¹⁸⁾

In conclusion, Hz and AcHz are cytotoxic to isolated hepatocytes and the initial stage of the toxic response appears to involve damage to the cell membrane, as described by Siemens *et al.*¹⁹⁾ The experiments using the hepatocyte system probably mimic quite well the *in vivo* characteristics of Hz toxicity in the liver.

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References

- 1) A. Noda, T. Sendo, K. Ohno, M. Misaka, Y. Kanazawa, R. Isobe, S. Goto, M. Ishizawa, H. Noda and M. Hirata, Abstracts of Papers, 17th Annual Symposium on Drug Metabolism and Action, Sendai, October 1985, p. 131.
- 2) J. Balo, *Adv. Cancer Res.*, **30**, 151 (1979).
- 3) A. V. Wright and L. Tikkanen, *Mutat. Res.*, **78**, 17 (1980).
- 4) A. Noda, T. Goromaru, K. Matsuyama, K. Sogabe, K.-Y. Hsu and S. Iguchi, *J. Pharmacobio-Dyn.*, **1**, 132 (1978).
- 5) J. A. Timbrell, J. R. Mitchell, W. R. Snodgrass and S. D. Nelson, *J. Pharmacol. Exp. Ther.*, **213**, 364 (1980).
- 6) A. Noda, K.-Y. Hsu, H. Noda, Y. Yamamoto and T. Kurozumi, *J. UOEH (Sangyo Idai Zasshi)*, **5**, 183 (1983).

- 7) A. Noda, unpublished data.
- 8) A. Noda, T. Sendo, K. Ohno, S. Goto, H. Noda and K-Y. Hsu, *Toxicol. Lett.*, **25**, 313 (1985).
- 9) T. Sendo, A. Noda, K. Ohno, S. Goto and H. Noda, *Chem. Pharm. Bull.*, **32**, 795 (1984).
- 10) S. D. Nelson and W. P. Gordon, *Adv. Exp. Med. Biol.*, **136B**, 971 (1981); D. L. Springer, D. J. Read and F. N. Dost, *J. Toxicol. Env. Health*, **8**, 11 (1981).
- 11) A. Noda, H. Noda, K. Ohno, T. Sendo, A. Misaka, Y. Kanazawa, R. Isobe and M. Hirata, *Biochem. Biophys. Res. Commun.*, **133**, 1086 (1985).
- 12) A. Noda, M. Ishizawa, K. Ohno, T. Sendo and H. Noda, *Toxicol. Lett.*, **31**, 33 (1986).
- 13) A. Noda, K-Y. Hsu, Y. Aso, K. Matsuyama and S. Iguchi, *J. Chromatogr.*, **230**, 345 (1982).
- 14) H. Baur, S. Kasperek and E. Pfaff, *Z. Physiol. Chem.*, **356**, 827 (1975).
- 15) M. Watanabe, K. Tonda, M. Hirata and Y. Hata, *Biochem. Biophys. Res. Commun.*, **112**, 356 (1983).
- 16) A. Piriou, A. Tacquesson, J. M. Warnet and J. R. Claude, *Toxicol. Lett.*, **17**, 301 (1983).
- 17) A. Misaka, A. Noda, S. Goto, Y. Kanazawa and H. Noda, Abstracts of Papers, 106th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, April 1986, p. 529. The details will be reported elsewhere.
- 18) R. C. James and R. D. Harbison, *Biochem. Pharmacol.*, **31**, 1829 (1982).
- 19) A. N. Siemens, M. C. Kitzes and M. W. Berns, *Toxicol. Appl. Pharmacol.*, **55**, 378 (1980).