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CELLULAR GLUTATHIONE CONJUGATION OF AZIRIDINES IN ISOLATED RAT HEPATOCYTES

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Abstract—Glutathione conjugation of aziridines was found in isolated rat hepatocytes in experiments using the optical isomers of (l- and d-) aziridinecarboxylic acid (AZC) and (l- and d-) 1-methyl-2-β-naphthylaziridine (NAZ). l-AZC much more effectively consumed glutathione than d-AZC, and the yield of the glutathione conjugate during 2 hr of incubation exceeded 200% of the cellular glutathione detected at the initiation of the incubation. Such a high yield of l-AZC–GSH conjugate would occur only when conjugation efficiently proceeds without interference against the GSH resynthesis route in the liver cells. The cytotoxicity of l-AZC was very weak and did not affect cell viability of the isolated hepatocytes even after the formation of AZC–GSH conjugate. Consequently, we supposed that GSH is not essential for supporting the viability of the isolated hepatocytes. For very slow GSH conjugate formation of d-AZC, we envisaged poor membrane transport of the d-isomer resembling to the selective incorporation of d- and l-proline observed in some plant cells.

Both isomers of NAZ were markedly cytotoxic and depressed the cell viability. The yield of the glutathione conjugate from NAZ did not exceed the cellular GSH level detected at the initial stage of incubation. The highly cytotoxic compound nitrosomethane, generated in the first biotransformation step of the metabolism of NAZ, can obstruct the resynthesis route of GSH by inhibiting the ATP generation process as discussed previously (Ref. 3). Decreasing the cellular GSH by treatment with l-AZC enhanced the susceptibility of the isolated hepatocytes to NAZ toxicity. d-AZC did not affect the viability of cells treated with NAZ.

Much of the new information about glutathione (GSH)† has come from studies on the reaction mechanism of toxic substances and cellular GSH. Cellular GSH has been found to control the homeostasis of calcium ion metabolism in living cells and its disorder would lead to cell death [1].

On the other hand, aziridine derivatives are used extensively as antitumor agents. Carbazilquinone and mitomycin C are especially widely used. Recently, aziridinecarboxylic acid derivatives have also been reported as effective immunomodulators [2]; however, the toxicity of aziridines remains as an ambiguous problem. Thus, we have studied the cytotoxicity of optically active 2-β-naphthylaziridines (d- and l-NAZ) and aziridinecarboxylic acids (d- and l-AZC) against hepatocytes isolated from rat [3]. In the investigation, we found that l-AZC formed the glutathione–aziridine conjugate in more than 200% yield, calculated on the basis of the amount of cellular GSH detected at the initial stage of the experiment. Surprisingly, cells with depleted GSH had the same life span as the controls. However, d- or l-NAZ formed only equimolar GSH-conjugate and caused death of the isolated hepatocytes. Here we report the reaction between aziridines and cellular GSH and discuss the viability of isolated hepatocytes with depleted cellular GSH.

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† Abbreviations used: NAZ, 1-methyl-2-β-naphthylaziridine; AZC, 2-aziridinecarboxylic acid; AZC–cysteine, *S*-(β-alanine-2-yl)cysteine; NAZ–cysteine, *S*-(1-β-naphthyl-2-methylaminoethyl)cysteine; AZC–GSH, *S*-(β-alanine-2-(*S*)-yl glutathione; NAZ–GSH, 1-*S*-(1-β-naphthyl-2-methylaminoethyl)glutathione; GSH, glutathione.

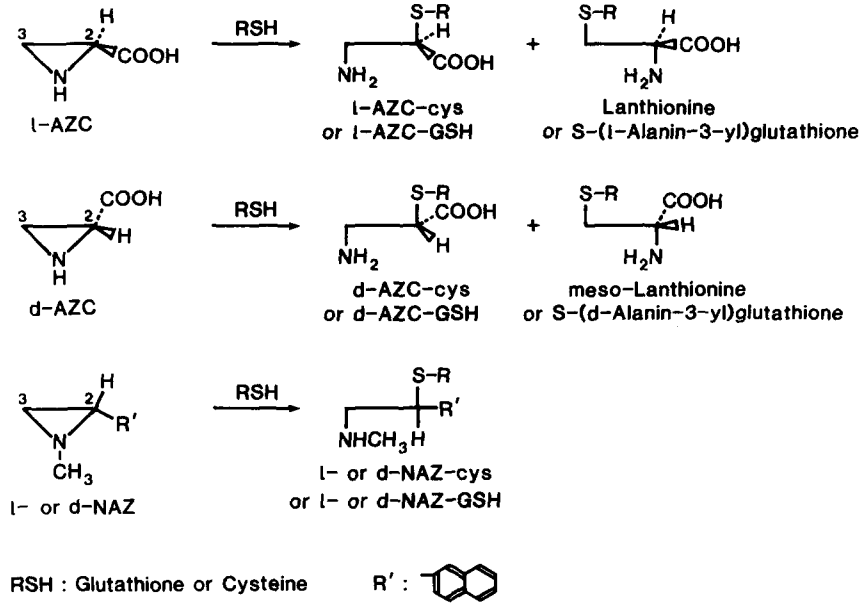
MATERIALS AND METHODS

Chemicals. l-Lanthionine and *meso*-lanthionine were gifts from Professor T. Shiba of Osaka University. l-NAZ, d-NAZ and lithium salts of l-AZC and d-AZC were prepared as described in the preceding report [3]. The lithium salt of AZC was converted to free acid immediately after it was added to the buffer solution. The absolute configurations of l- and d-AZC are shown in the figures.

Analytical procedures. AZC–cysteine or AZC–GSH conjugates were chromatographed on a TSK-GEL SP-2SW (0.4 × 25 cm) column with 5% CH_3CN 50 mM phosphate buffer as a mobile phase at pH 2.65 for AZC–cysteine or at pH 3.0 for AZC–GSH and detected by fluorimetry [4]. NAZ–cysteine or NAZ–GSH conjugates were also separated by HPLC using Nucleosil 5-C_{18} (0.4 × 25 cm) column with 10% CH_3CN-50 mM phosphate buffer, pH 7.0, as the mobile phase and detected at 254 nm. Cellular GSH was determined colorimetrically according to Saville [5]. Cellular ATP was determined by HPLC as described previously [3].

The structures of AZC–GSH, NAZ–GSH, AZC–cysteine or NAZ–cysteine were determined by comparison with authentic samples [6].

Incubation of aziridines in isolated hepatocytes suspension. Parenchymal cells were isolated from male Wistar rats (300–330 g), fed *ad libitum* by collagenase perfusion [7, 8]. Cell viability was judged to be 97–98% from the LDH latency test [9]. Aziridines (0.5 mM) were incubated with 3×10^6 cells/ml isolated hepatocytes in Krebs–Henseleit buffer, pH 7.4, containing 1% bovine serum albumin, 10 ml glucose, 13 mM Hepes, penicillin and amino acid mixture



Scheme 1

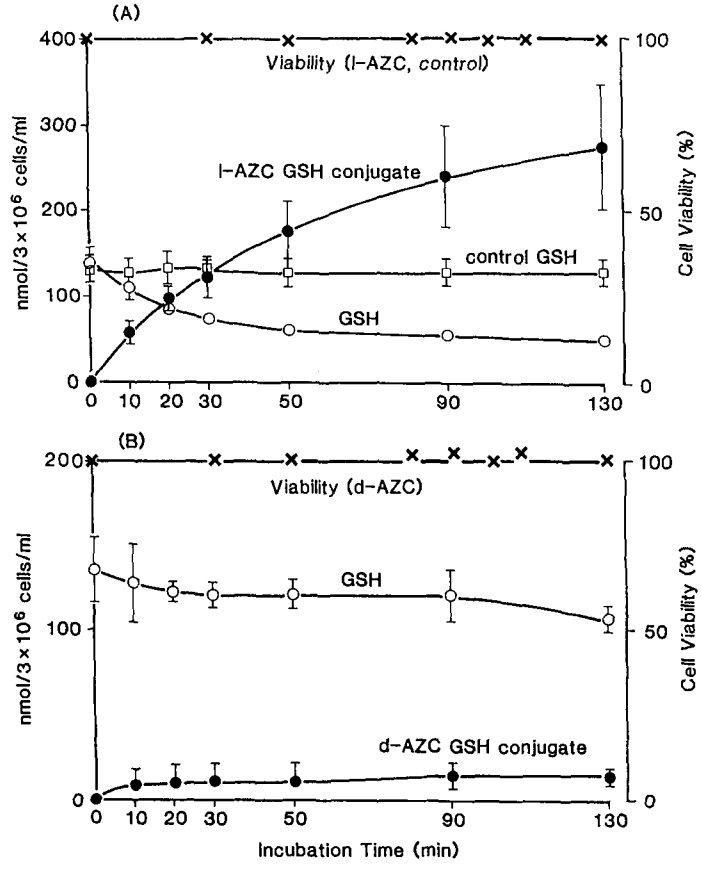


Fig. 1. GSH conjugation of l- and d-AZC in isolated hepatocytes. AZC in 0.5 mM was incubated 37° with 3 × 10^6 cells/ml in Krebs–Henseleit buffer, pH 7.4, supplemented with 1% BSA and other additives as described in the text. (A) Cellular GSH as a control (□—□), GSH remaining in the incubation mixture (○—○), and l-AZC—GSH formed (●—●). The values indicate mean ± SE for three experiments. Viability of the isolated hepatocytes (×—×) was cited from Ref. 3.

(Gibco) at 37° in a rotating round-bottom flask under an atmosphere of O_2–CO_2 (95:5). At intervals, a portion was transferred to a test-tube together with an equal volume of 0.5 N $HClO_4$. After centrifugation for 10 min at 3000 rpm, the supernatant was chromatographed on an HPLC column. The eluate was hydrolyzed with 6 N HCl and analyzed as described above. The aziridine–GSH conjugates obtained were confirmed to be only those resulting from GSH attack of the C-2 position of aziridines. l-Lanchionine was expected from the hydrolysis of *S*-(1-alanin-3-yl)glutathione which formed by the attack of GSH on the C-3 atom of l-AZC. This compound formed in the phosphate buffer solution but was not found in the hepatocyte suspension at the limit of 0.1% of the yield. The results obtained are shown in Figs. 1 and 2.

The viability of the isolated hepatocytes treated with 0.5 mM of l-NAZ was studied according to the procedure described in Ref. [3]. The experiments using both AZC and NAZ were carried out by treatment with 0.5 mM AZC solution 10 min before the addition of NAZ. Study of the viability was started after addition of NAZ. The results are shown in Fig. 3.

Incubation of aziridines in rat liver cytosol. Rat liver was excised, after infusion with 0.9% NaCl under ether anesthesia, and homogenized with five volumes of ice-chilled 50 mM phosphate buffer, pH 7.4, containing 3 mM GSH. The homogenate was centrifuged first at 2000 rpm for 10 min then at 40,000 rpm for 60 min at 4°. The supernatant obtained was diluted with 50 mM phosphate buffer, pH 7.4, to obtain the appropriate protein concentration. GSH (3.06 mg/ml) was added to the freshly-prepared extract, then aziridine (final conc. 1 mM) to start the reaction. The mixture was incubated at 37° with mild shaking and a portion of the incubation mixture was transferred to a tube with an equal amount of 0.5 N $HClO_4$ followed by centrifugation for 2 min at 10,000 rpm. Aziridine–GSH conjugates were separated by HPLC, freeze-dried, hydrolyzed with 6 N HCl for 24 hr at 110° to the corresponding aziridine–cysteine, and measured. AZC–GSH conjugate formation for d- and l-AZC occurred at the same rate.

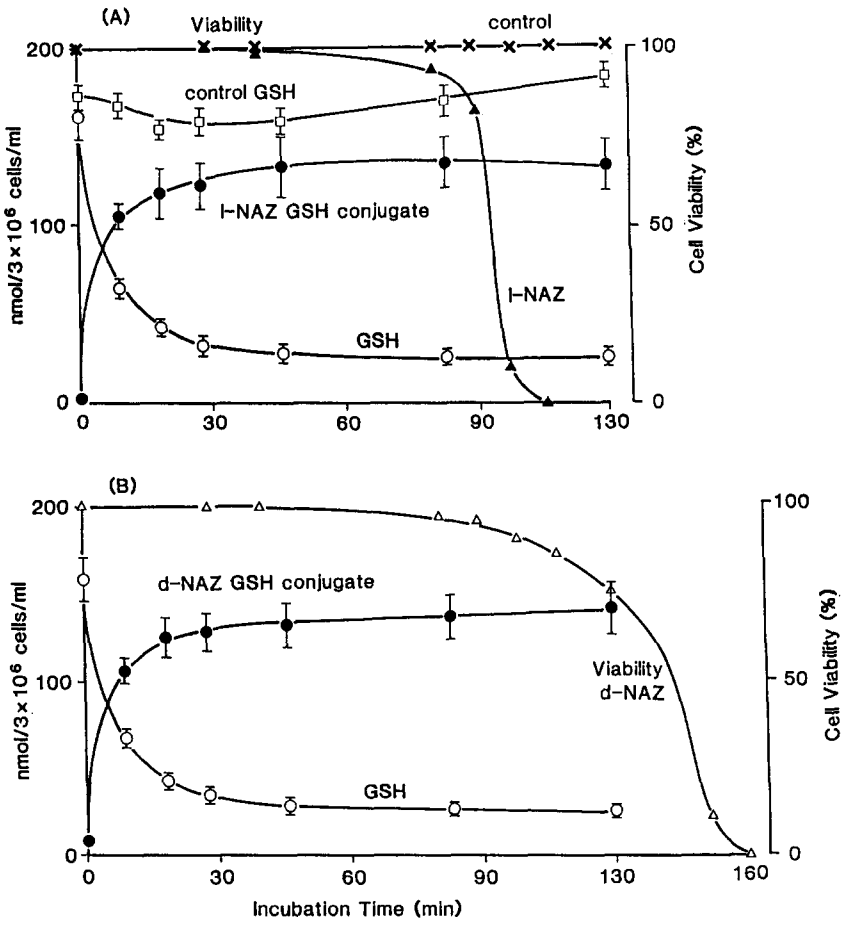


Fig. 2. Conjugation of l- and d-NAZ and GSH in isolated hepatocytes. Isolated rat hepatocytes were incubated with 0.5 mM NAZ as described in the text. (A) Cellular GSH in control experiment (□—□), GSH remaining within cells (○—○), l-NAZ–GSH conjugate (●—●), viability of the isolated hepatocytes (▲—▲) and control for experiment of viability (×—×). (B) GSH remaining (○—○), d-NAZ–GSH formed (●—●). The values indicate mean ± SE of three experiments. Viability of the isolated hepatocytes (△—△) cited from Ref. 3. Control for viability was the same as (A).

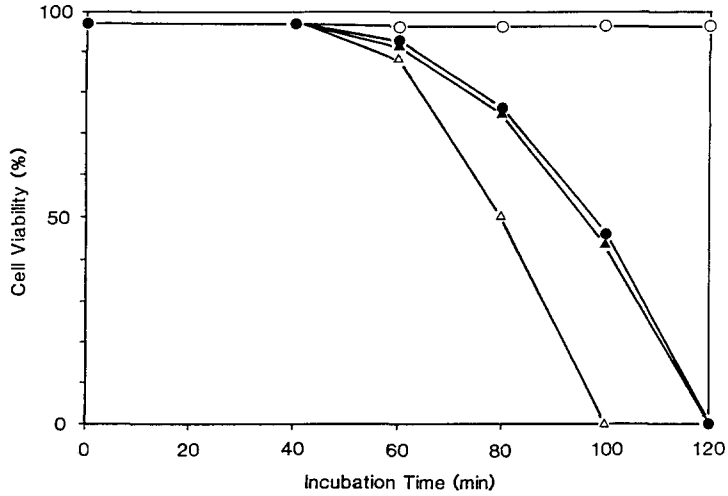


Fig. 3. Effects of l- and d-AZC on the l-NAZ cytotoxicity of isolated hepatocytes. Isolated hepatocytes were treated with 0.5 mM of l- or d-AZC at 10 min before the addition of l-NAZ. l-AZC caused enhancement of cell susceptibility to l-NAZ cytotoxicity by the consumed cellular GSH. d-AZC was independent of the cytotoxicity of l-NAZ based on the meaning described in the text. Cell viability was calculated after the addition of l-NAZ. Treatment with l-NAZ alone (●—●), l-AZC at 10 min before l-NAZ (△—△) and d-AZC before l-NAZ (▲—▲). The results of one typical experiment of five are shown. Viability values ranged between 0.8- and 1.2-fold depending on the rat from which the hepatocytes had been isolated. Consequently, SE calculation would have been meaningless and we used the most common value in the figure.

Aziridine–GSH conjugate formation in Krebs–Henseleit buffer containing bovine serum albumin. To study the absorption of aziridine on albumin, l- and d-AZC were incubated in Krebs–Henseleit buffer containing 1% bovine serum albumin and other additives at 37° for 30 min and protein binding was examined by ultrafiltration using Molcut SjGC (Millipore Ltd.). Although approximately 15 and 35% of AZC became bound to the albumin at 0.5 mM and 0.1 mM of AZC concentration, respectively, no difference between d- and l-isomers of AZC was observed.

RESULTS AND DISCUSSION

A very interesting feature of l-AZC metabolism in isolated hepatocytes was that the l-AZC–GSH conjugate formation exceeded 200% of the initial cellular GSH, with the viability of the isolated hepatocytes remaining the same as shown in Fig. 1. Thus, the life span of the isolated hepatocytes observed in the incubation solution was the same as that of the control experiment. This indicated clearly that GSH was not essential for retaining the viability of the isolated hepatocytes although there is much information that indicates glutathione depletion does not compromise cell viability [10]. Such a high yield of the l-AZC–GSH conjugate would be possible only when conjugation efficiently proceeds without interference of GSH resynthesis in the liver cells. In regard to this, l-AZC could serve as a useful tool for examining the rate of GSH synthesis within the cells.

As shown in Figs. 1 A and B, conjugation of d-AZC was much slower than that of l-AZC. Here, there are three possible explanations. The first assumption is that a permeability difference exists between d- and l-AZC for the cell wall of the isolated hepatocytes [11]. The second is that d-AZC is only slowly incorporated into the active center of GSH transferase [12, 13], and the third is the difference on the rate of the absorption ability of each of the AZC enantiomers on serum albumin [14, 15].

As described in Materials and Methods, the use of rat liver cytosol gave the same amounts of l-AZC–GSH and d-AZC–GSH whereas the intact cells inhibited the formation of the latter conjugate. Clearly the results suggested that the slower formation of d-AZC–GSH than of l-AZC–GSH, shown in Figs. 1 A and B, was due to poor membrane transport of the d-isomer [16]. Thus, the first assumption described above was reasonable, but not the second one. On the other hand, the incubation of AZC in Krebs–Henseleit buffer with bovine serum albumin denied the third assumption by having no effect on the formation of GSH conjugates of the enantiomers of AZC. Here, AZC is a kind of α-amino acid having a secondary amino group and its structure resembles that of proline. Selective incorporation of d- and l-proline by murine cells or by some plant cells [17] support our conclusion of slow d-AZC–GSH formation in the isolated hepatocytes.

Figures 2 A and B show the d- or l-NAZ–GSH conjugate formation and the viability of isolated hepatocytes treated with d- or l-NAZ, respectively. In contrast to l-AZC, the amount of d- or l-NAZ–GSH conjugate did not exceed the amount of the initial cellular GSH and their viability was also less.

Previously, we have reported that the strong cytotoxicity of d- and l-NAZ against isolated hepatocytes is caused by the formation of nitrosomethane via

decomposition of NAZ by cellular amine-oxidases [3, 18]. The nitrosomethane generated in the cells decomposed the ATP-generating system [3]. In the experiments of Figs. 2 A and B, nitrosomethane formed concurrently with NAZ–GSH conjugate formation and it should have inhibited the resynthesis cycle of cellular GSH.

The isolated hepatocytes which had been treated with l-AZC showed higher susceptibility to l-NAZ cytotoxicity than those treated with l-NAZ. However, earlier treatment with d-AZC, as shown in Fig. 3, did not affect the susceptibility of the isolated hepatocytes to l-NAZ toxicity. This is understandable because the l-AZC consumed cellular GSH of isolated hepatocytes by forming l-AZC–GSH and making it advantageous for nitrosomethane to be produced from NAZ. The consumption of d-AZC was independent of the cellular GSH.

CONCLUSION

As discussed in the Introduction, glutathione is considered to be an indispensable material for living animal cells. However, our studies showed that even marked depletion of GSH in isolated hepatocytes did not affect cellular viability. Cells which had been treated earlier with l-AZC showed increased susceptibility to cytotoxic compounds such as NAZ.

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