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DEMONSTRATION OF ENZYMATIC ACTIVITY CONVERTING AZATHIOPRINE TO 6-MERCAPTOPURINE

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Abstract. The enzymatic conversion of azathioprine to 6-mercaptopurine was detected at pH 6.5 with rat liver supernatants, although the non-enzymatic reaction predominated at pH 7.0 and 7.5. Glutathione S-transferase may catalize this conversion. Activities of the enzyme in liver with both azathioprine and 1, 2-dichloro-4-nitrobenzene as substrate decreased upon carbon tetrachloride-induced hepatic injury. These results may explain an ineffectiveness of azathioprine in patients with severe hepatic damage.

Key words: azathioprine, 6-mercaptopurine, glutathione S-transferase, rat liver

Azathioprine is known to be non-enzymatically degraded to 6-mercaptopurine (6-MP) by sulfhydryl compounds *in vitro* (1). After the administration of azathioprine *in vivo*, the recovery of significant amounts of 5-glutathionyl-1-methyl-4-nitroimidazole demonstrates that reduced glutathione (GSH)-mediated thiolysis is the major pathway for its conversion to 6-MP (2). It is not clear, however, where this conversion mainly occurs *in vivo*, why azathioprine is ineffective in the treatment of patients with severe liver damage (3) and how the metabolism of azathioprine is altered in injured liver (4). The conversion of azathioprine to 6-MP *in vivo* appears to be the first step in the sequence that leads to the biologically active drug (5). During the course of biochemical studies on azathioprine metabolism in damaged liver, an enzymatic reaction converting azathioprine to 6-MP was detected in rat liver supernatants. Recently Kaplowitz has also suggested similarly that the hepatic thiolysis of azathioprine mediated by GSH might be enzymatic *in vivo* (6). The inactivation of azathioprine as an immunosuppressive agent by pretreatment with sulfhydryl compounds such as cysteine or GSH may be due to a more rapid conversion of azathioprine into 6-MP before azathioprine penetrates the target cells (5). The first step of azathioprine metabolism is therefore very important in the sense that the chemotherapeutic advantages of azathioprine over 6-MP are well known (7). The purpose of this study is to define the site for the cellular uptake of azathioprine by measuring the acute

disappearance of GSH after the administration of the drug. The enzymatic conversion of azathioprine to 6-MP and the role of glutathione S-transferase in initiating azathioprine metabolism in the liver are also described.

MATERIALS AND METHODS

Materials. Azathioprine, 6-MP and GSH were obtained from Sigma Chemical Co., St. Louis, Mo. 1, 2-Dichloro-4-nitrobenzene was purchased from Ishizu Pharmaceutical Co., Ltd., Osaka. Bromosulfophtalein (BSP) was kindly supplied by Daiichi-Pharmaceutical Co., Tokyo.

Animals. Male Sprague-Dawley rats weighing 180g were used throughout the present study. Animals were starved overnight prior to the experiments. Carbon tetrachloride (CCl_4) was administered by intragastric intubation in a single dose of 0.5ml of a 20% solution in liquid paraffin per 100g body weight and given water *ad libitum* until sacrifice. Rats were given sodium phenobarbital intraperitoneally at a dose of 8 mg/100g body weight daily for 3 days. Control animals were starved overnight.

Analytical procedures. Liver supernatants in 4 volumes of 0.1 M phosphate buffer, pH 7.4, were prepared as previously reported (8) and used as an enzyme source. Glutathione S-transferase activity was measured by using 1mM 1, 2-dichloro-4-nitrobenzene and 4.5mM GSH as substrate at 37°C and a wavelength of 345 nm in a final volume of 1.0ml (9). The molar absorption of S-chloronitrophenyl glutathione used was 8500/cm (9).

A cuvette containing all the constituents of the following reaction mixture except liver supernatant was used as a blank. The rate of increase in absorbance at 325nm was used as a measure of the rate of 6-MP formation, non-enzymatic conversion of azathioprine to 6-MP being obtained from the blank (7). The reaction mixtures contained 4.5mM GSH, 0.1mM azathioprine, 0.1 M phosphate buffer, pH 6.5, 7.0 and 7.5, and liver supernatants as the enzyme source in a final volume of 1.0ml, unless otherwise indicated. The reaction was initiated by adding GSH at 37°C and read at 325nm, where near maximal absorbance of 6-MP was obtained at a molar absorbtion of 19300/cm at pH 6.5. Twenty five μl of liver supernatant used for assays contained about 0.03 μmole GSH, which is negligible as compared with the concentrations of GSH added as substrate. Under these conditions, the initial reaction rates were linear for at least 10min or within 0.05units change in absorbance/min. One unit of the enzyme activity was defined as the amount that led to the formation of 1 μmole of 6-MP/min. Specific activity is expressed in units of enzyme activity per mg of protein. Protein was estimated by the method of Lowry *et al.* (10). GSH contents of liver and blood were determined as reported previously (11).

RESULTS

A single intragastric administration of 500 mg/kg body weight of azathioprine resulted in a rapid fall of the hepatic GSH levels; GSH levels decreased to about 50% of the control levels from 30min to 2h after the treatment and were

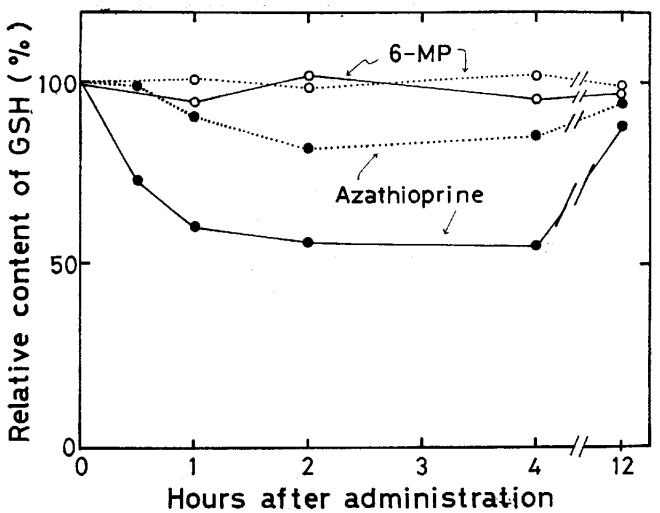

Fig. 1. GSH levels in liver and blood after the administration of azathioprine and 6-MP to rats. Azathioprine (●) and 6-MP (○) were given intragastrically to male Sprague-Dawley rats at a dose of 50 and 31 mg/100 g body weight, respectively. GSH contents in liver supernatants (——) and blood hemolysate (·····) were determined at the times indicated. The average values from 2 rats are shown.

restored approximately to the control values within 12 h (Fig. 1). No decrease of GSH content in blood was observed during the first 30 min after azathioprine administration, whereas a 20% drop of the GSH levels in blood hemolysate was observed from the 2nd to the 4th h after administration. On the other hand, GSH levels both in the circulating blood and livers remained unchanged even when an equimolar dose of 6-MP was administered. A dose of 50 mg/kg body weight of azathioprine given intragastrically to rats resulted in no significant change in GSH levels in liver. No significant alteration in GSH concentration of kidney was ever found even with the dose of 500 mg/kg body weight during the experiments. Non-enzymatic formation of 6-MP from azathioprine at 37° C and at pH 6.5 to 7.5 with constant concentrations of 0.1 mM azathioprine and 4.5 mM GSH is shown in Fig. 2. At alkaline pH, the conversion is mostly non-enzymatic, the reaction being dependent on the concentration of GSH (7). The conversion of azathioprine to 6-MP may be enzymatic at pH 6.5, since the increasing amounts of liver supernatants added to the assay mixture up to 25 μl produced a linear increase of the formation of 6-MP (Fig. 2). Since BSP has been reported to be a competitive inhibitor for glutathione S-transferase with 1, 2-dichloro-4-nitrobenzene as subtrate (9), inhibition of the enzymatic formation of 6-MP from azathioprine by 0.06 mM BSP was determined with 4.5 mM GSH. The Km for azathioprine was found to be 0.03 mM, which is much lower than the concentra-

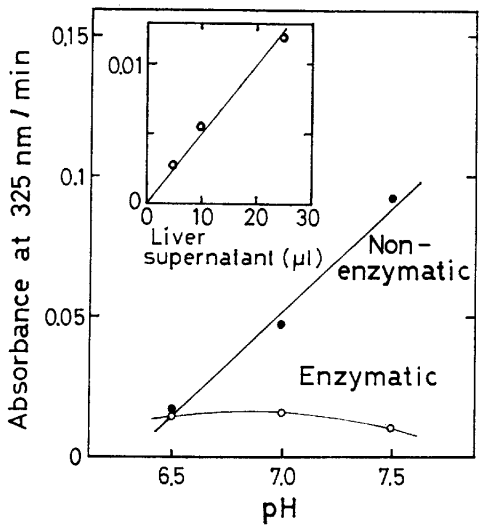

Fig. 2. Non-enzymatic and enzymatic conversions of azathioprine to 6-MP at various pH values. Buffers used were 0.1 M phosphate buffer at pH 6.5, 7.0 and 7.5. Activities of the enzymatic reaction using 25 μl liver supernatant (○) were plotted after subtracting for non-enzymatic changes. Non-enzymatic conversions (●) are determined in the absence of liver supernatants. Inset indicates the enzymatic conversion of azathioprine to 6-MP at pH 6.5 with the various volumes of liver supernatants.

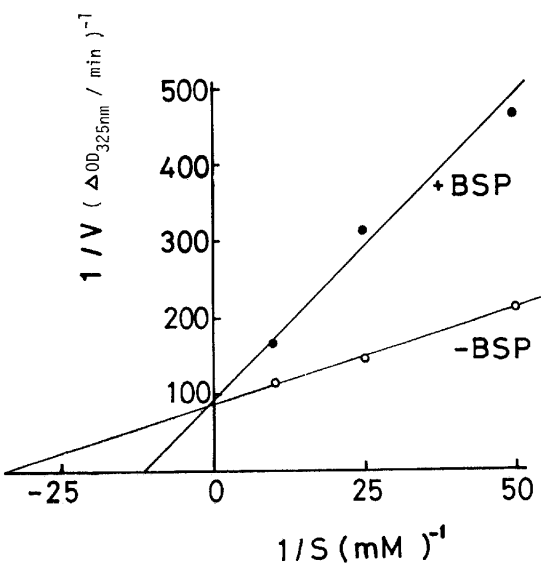

Fig. 3. A Lineweaver-Burk plot of the enzymatic conversion of azathioprine to 6-MP in the presence and absence of BSP. The concentration of BSP added was 0.06 mM. Azathioprine concentrations used as substrate are 0.1, 0.04 and 0.02 mM. Km is estimated to be 0.03 mM.

tion of azathioprine as substrate for the assay. BSP was also found to be a competitive inhibitor for the 6-MP-forming activity (Fig. 3). Increasing concentrations of BSP produced a marked inhibition of the enzyme activity with azathioprine as substrate, but 6-MP had no such effect. Similar inhibitory effects of BSP were observed with 1, 2 dichloro-4-nitrobenzene as substrate (Fig. 4).

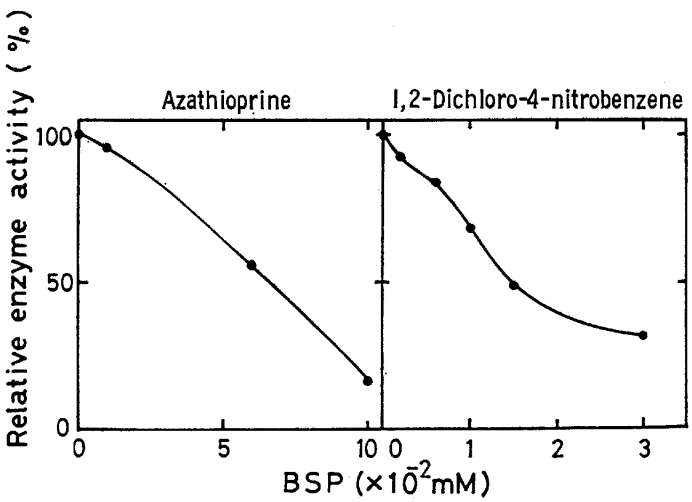

Fig. 4. Inhibition of the enzymatic conversion of azathioprine to 6-MP by BSP. The assay system was the complete assay mixture with azathioprine and 1, 2-dichloro-4-nitrobenzene as substrate (See Materials and Methods). The relative enzyme activities were shown as percent.

The values of the enzymatic activity for azathioprine in liver are consistent to those previously reported under normal conditions (6). In CCl_4-treated rats, glutathione S-transferase activities with 1, 2-dichloro-4-nitrobenzene were observed to decrease in the supernatant fractions from injured livers. Activities with azathioprine as substrate were similarly found to decrease upon the treatment (Table 1). An addition of CCl_4 *in vitro* gave no direct effect on the enzyme

TABLE 1. GLUTATHIONE S-TRANSFERASE ACTIVITY IN PHENOBARBITAL AND CCL_4-TREATED RAT LIVER

Treatment (No. of rats)	Glutathione S-transferase (mU/mg protein)	
	1, 2-Dichloro-4-nitrobenzene	Azathioprine
None (3)	63.6±2.1	1.3±0.1
Phenobarbital (3)	70.1±5.8	1.3±0.1
CCl_4 (4)	24.5±4.9*	0.8±0.1*

*, $P<0.05$

reaction. The enzyme activity with azathioprine as substrate could be shown to be present also in kidney and spleen, however, the activity was very low. Only negligible activity was observed in other tissues, the distribution being quite similar to that of glutathione S-transferase (9). Rats treated with phenobarbital daily for 3 days showed no induction of the enzymatic reaction for azathioprine and 1, 2-dichloro-4-nitrobenzene as substrate. These data are not consistent to the previous observation (12), although long-term treatment with phenobarbital was described in that report.

DISCUSSION

The disappearance of GSH seems to be a result of its covalent conjugation with the imidazole moiety of azathioprine and can be used as an indicator for the intracellular thiolysis of azathioprine. The marked decrease of GSH content in liver and the moderate decrease in blood after azathioprine administration, which seem to be in a dose-related fashion, suggest that liver and probably blood are major sites for the metabolism of azathioprine to 6-MP.

This report also revealed the presence of the enzymatic reaction converting azathioprine to 6-MP with liver supernatants at pH 6.5. BSP is a competitive inhibitor of azathioprine conjugation with GSH, and the enzyme activities with azathioprine and 1, 2-dichloro-4-nitrobenzene as substrate similarly decreased upon CCl_4 treatment. These results seem to suggest that glutathione S-transferase catalyzes this conversion with broad substrate specificity. Kaplowitz (5) has suggested from the data on elution in gel filtration, inhibition kinetics using bilirubin and the organ distribution of this enzyme that the conversion of azathioprine to 6-MP is catalyzed by glutathione S-transferase at least *in vitro*. Although drug-metabolizing enzymes depending upon cytochrome P-450 are located mainly in liver microsomes, many other drugs are metabolized also in the liver supernatant (13).

As both the enzyme activity converting azathioprine to 6-MP and hepatic GSH levels are decreased in CCl_4-damaged livers (11), this conversion may be impaired under these conditions. However, in view of the small proportion of the enzymatic conversion at physiological pH, it is not clear that this impairment is of clinical importance. We are now measuring the blood clearance of azathioprine to investigate whether the impaired conversion to 6-MP could be observed in patients with various liver diseases or not. Impaired activation of azathioprine in liver diseases might be due to reduced hepatic uptake of azathioprine, decreased GSH and glutathione S-transferase levels and inhibition of this enzyme by raised bilirubin concentrations. If the conversion of azathioprine to 6-MP *in vivo* is exclusively chemical, it may be inferred that, in severe liver diseases, the activity of hypoxanthine-guanine phosphoribosyltransferase is decreased and there

is excessive catabolism of 6-MP or thioinosinic acid. It is also very important in clinical pharmacology to see whether simultaneous injections of GSH to systematically azathioprine-treated patients are useful in preventing liver injury as a side effect or increasing the intracellular levels of active azathioprine metabolites in the target tissues, namely the immunocytes.

Further studies are needed to define the role *in vivo* of glutathione S-transferase in the conversion of azathioprine to 6-MP in order to answer the question of whether the conversion in liver cells is enzymatic, non-enzymatic or both.

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