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PRELIMINARY COMMUNICATION

ENZYMATIC THIOLYSIS OF AZATHIOPRINE IN VITRO

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Azathioprine undergoes thiolysis to 6-mercaptopurine in vivo, a step necessary for the ultimate action of the drug [1,2]. It has long been suspected that the cleavage of azathioprine at the S-imidazole bond involves thiol compounds, particularly glutathione [3]. After the administration of azathioprine in vivo, the recovery of significant amounts of 5-glutathionyl-1-methyl-4-nitroimidazole [4,5] demonstrates that glutathione-mediated thiolysis is the major pathway for conversion to 6-mercaptopurine. Previous evidence obtained in vitro has favored nonenzymatic thiolysis of azathioprine by glutathione of liver and erythrocytes [4-6]. The failure of azathioprine to produce plasma rosette inhibitory activity in liver disease [7-9] suggests that hepatic metabolism of azathioprine is necessary for activation of the drug. Therefore, studies were performed using rat liver to identify the presence of an enzyme activity capable of catalyzing the thiolysis of azathioprine by glutathione.

6-Mercaptopurine was found to have λ_{max} at 317 nm whereas azathioprine had a λ_{max} at 280 nm in 0.1 M sodium phosphate buffer, pH 6.5 to 8.0, at 37°. The molar extinction coefficient for 6-mercaptopurine was 15.2 mM^{-1} cm^{-1}. After complete thiolysis (2-hr incubation at 37°) of azathioprine by glutathione with or without added liver homogenate, the identical molar extinction coefficient was derived for the product of the reaction, confirming that 6-mercaptopurine is a product of the interaction between azathioprine and glutathione.

The existence of an hepatic enzyme activity for the reaction between azathioprine and glutathione and the production of 6-mercaptopurine (at 317 nm) was sought (Table 1). The 100,000 g supernatant fraction (cytosol) from male Sprague-Dawley rat liver (20% homogenate) was prepared as previously described [10]. Reaction mixtures (2.1 ml) contained azathioprine (0.1 mM) in 0.1 M sodium phosphate buffer, pH 6.5 to 8.0. Reaction was initiated by the addition of glutathione (0.5 mM) with or without 100 µl liver cytosol. Reactions were followed spectrophotometrically for the production of the 317 nm product in a Beckman ACTA MVI spectrophotometer (0 to 0.1 absorbance scale) at 37° with the reference consisting of all the constituents of the reaction mixture except azathioprine. When cytosol was used a total activity was measured, whereas in the absence of cytosol a nonenzymatic rate was measured. The difference represented enzymatic activity. An enzymatic activity (linear for 5 min) was found which was a linear function of protein and substrate concentration at each pH tested and which was destroyed by heating cytosol at 100° for 5 min. The predominance of the nonenzymatic activity at high pH values has been previously described [3]. The predominance of the enzymatic reaction at lower pH values that prevail

in normal cytoplasm suggests that a function of the enzyme is to effect the ionization of glutathione, thereby promoting nucleophilic attack on the electrophilic site of the imidazole moiety of azathioprine. This presumed lowering of the pKa of glutathione is believed to be fundamental to the catalytic properties of a group of hepatic cytosolic enzymes, the glutathione S-transferases [11,12].

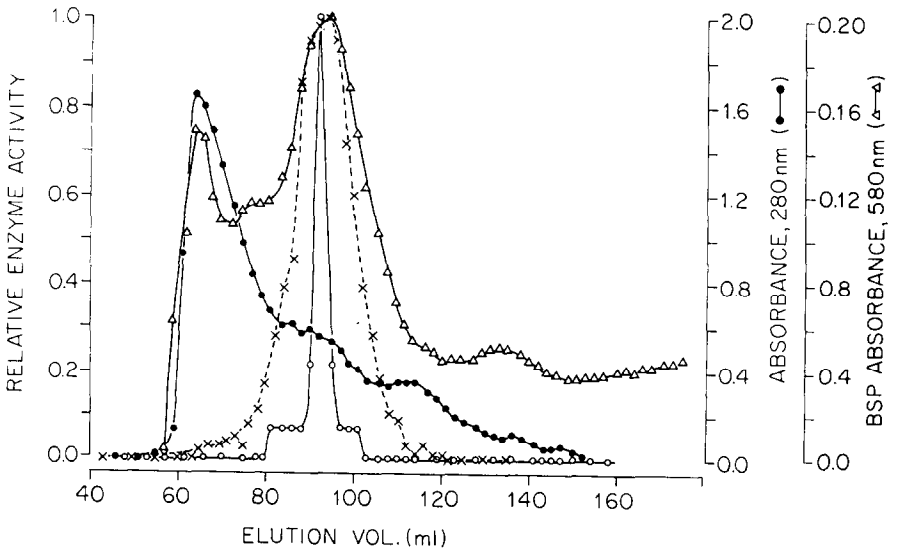
Table 1. Effect of pH on the interaction of azathioprine and glutathione

	Activity (nm min^{-1})			
pH	Total	Non-enzymatic	Enzymatic	% Enzymatic
6.5	2.5	0.5	2.0	80
7.0	2.8	0.8	2.0	72
7.4	3.4	1.8	1.6	47
8.0	4.3	3.7	0.6	14

An attempt was made to relate the soluble hepatic enzyme activity for azathioprine and glutathione to the glutathione S-transferases. Four characteristics of the latter were studied: (1) elution in gel filtration, (2) inhibitory kinetics by bilirubin and probenecid, (3) induction by phenobarbital, and (4) organ distribution in the rat.

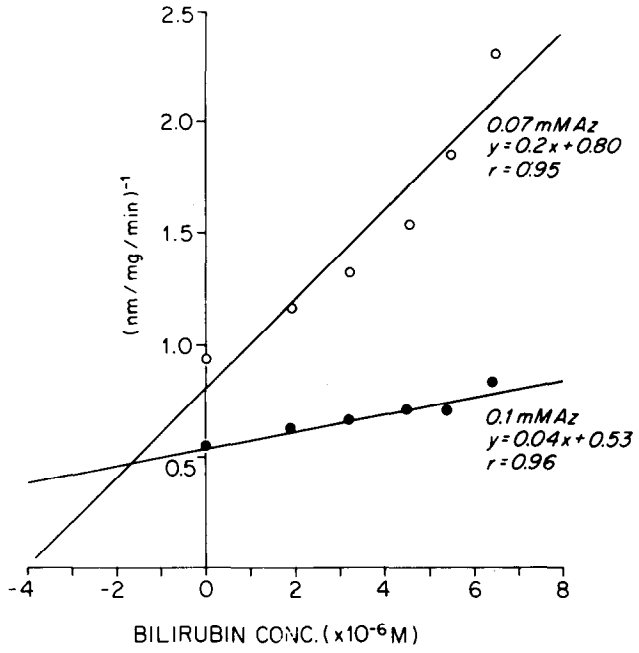
Gel filtration was performed using Sephadex G-100 (38 cm x 2.5 cm column) and a pump-driven upward flow system with 0.01 M phosphate buffer, pH 7.4, as the mobile phase (flow rate 24 ml/hr; ten fractions collected/hr). The column was loaded with 3.0 ml liver cytosol to which was added 1.0 mg sulfobromophthalein immediately prior to loading. Column fractions were assayed for the following: protein (280 nm), sulfobromophthalein binding (580 nm following alkalinization), enzyme activity for azathioprine and for 1-chloro-2,4-dinitrobenzene, a representative substrate for the glutathione S-transferases [11]. The elution profile for sulfobromophthalein binding, the glutathione S-transferase activity and azathioprine activity were identical (Fig. 1). This indicates in a crude fashion that the molecular weight of the azathioprine activity and the transferases is closely similar. Moreover, this is further supported by the correspondence of sulfobromophthalein binding protein(s) and the azathioprine activity. Sulfobromophthalein is known to bind to Y protein or ligandin [13]. The latter has been shown to be one of the glutathione S-transferases [14].

Fig. 1. Comparison of the elution of azathioprine thiolytic activity, glutathione S-transferase activity, and sulfobromophthalein binding in the gel filtration of liver cytosol. Azathioprine (-O-) and 1-chloro-2,4-dinitrobenzene (-X-) activities for convenience are expressed as a ratio to the maximum activity in the column fractions.



The glutathione S-transferases bind compounds which are not substrates for these enzymes, such as bilirubin and probenecid [10,14,15]. One means by which bilirubin and probenecid have been shown to bind to the glutathione S-transferases has been the demonstrations that these compounds are competitive inhibitors of these enzymes [10,15]. Therefore, these two compounds were studied as inhibitors of the hepatic enzyme activity for azathioprine. Assay conditions were as described above and kinetics were studied as previously described [10]. A Dixon plot [16] of the competitive inhibition by bilirubin of the azathioprine activity of liver cytosol is shown in Fig. 2 (K_i = 1.6 μM). Similarly probenecid was found to be a competitive inhibitor of this enzymatic reaction (K_i = 27 μM).

Fig. 2. Dixon plot showing the competitive inhibition by bilirubin of the enzymatic thiolysis of azathioprine by glutathione. Az indicates azathioprine. The equations for the lines were calculated by least squares regression and the inhibitor constant (K_i) was calculated from these equations.



Rats (200 g male Sprague-Dawley) were given sodium phenobarbital (80 mg/g, i.p.) daily for 10 days after which the animals were killed and liver cytosol was prepared. Groups of four treatment and control animals were studied. As indicated in Table 2 which shows kinetic data derived from a Lineweaver-Burk analysis [17], induction of the enzymatic activity for azathioprine was observed: significantly increased V_{max} with no change in apparent K_m. This induction paralleled the previously reported induction by phenobarbital of hepatic glutathione S-transferases [18].

Table 2. Phenobarbital induction of enzymatic thiolysis of azathioprine

Treatment	K_m (mM)	V_{max} (nm min^{-1} mg^{-1})
Control (saline)	0.24 ± 0.06	4.0 ± 0.6
Phenobarbital	0.24 ± 0.04	8.3 ± 1.1*

*P < 0.02 vs control.

The glutathione S-transferases have been identified in kidney [19] and small intestine [20] as well as liver [18]. Azathioprine enzymatic activity was measured as above at pH 6.5, 37° using 0.1 mM azathioprine, 0.5 mM glutathione, and 100 µl of the cytosolic fraction from a 20% homogenate of these organs from four rats. The following levels expressed as specific activities per mg of cytosolic protein were found: liver 1.1 ± 0.02, small intestine 0.48 ± 0.16 and kidney 0.17 ± 0.02 (nm min^{-1} mg^{-1}).

We have identified an enzyme activity which catalyzes the thiolysis of azathioprine by glutathione to produce 6-mercaptopurine. The striking similarities of this activity to the glutathione S-transferases include molecular weight in gel filtration, inhibitory kinetics with probenecid and bilirubin, induction by phenobarbital, and organ distribution. Further work with purified glutathione S-transferases is needed to establish for which of the closely related individual enzymes azathioprine is a substrate. In addition, studies are needed to define the role *in vivo* of this enzyme activity in the conversion of azathioprine to 6-mercaptopurine. It is tempting to speculate that impaired activation of azathioprine in liver disease could be the result of reduced hepatic conversion of azathioprine to 6-mercaptopurine as a consequence of one or more of the following possibilities: reduced hepatic uptake of azathioprine, reduced levels of hepatic thiolytic enzyme activity, reduced levels of glutathione, or inhibition of enzymatic thiolysis of azathioprine by raised intracytoplasmic levels of bilirubin or other compounds.

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REFERENCES

1. G. Elion, S. Callahan, S. Bieber, G. Hitchings and R. Rundles, Cancer Chemother. Rep. 14, 93 (1961).
2. G. Elion, Proc. R. Soc. Med. 65, 257 (1972).
3. A. Chalmers, P. Knight and M. Atkinson, Aust. J. exp. Biol. med. Sci. 45, 681 (1967).
4. A. Chalmers, Biochem. Pharmac. 23, 1891 (1974).
5. P. de Miranda, L. Beacham, T. Creagh and G. Elion, J. Pharmac. exp. Ther. 195, 50 (1975).
6. P. de Miranda, L. Beacham, T. Creagh and G. Elion, J. Pharmac. exp. Ther. 187, 588 (1973).
7. C. Mitchell, A. Eddleston, M. Smith and R. Williams, Lancet 1, 1196 (1970).
8. J. Bach and M. Dardenne, Proc. R. Soc. Med. 65, 260 (1972).
9. G. Whelan and S. Sherlock, Gut 13, 907 (1972).
10. N. Kaplowitz, J. Kuhlenkamp and G. Clifton, Proc. Soc. exp. Biol. Med. 149, 234 (1975).
11. W. Habig, M. Pabst and W. Jakoby, J. biol. Chem. 249, 7130 (1974).
12. M. Pabst, W. Habig and W. Jakoby, J. biol. Chem. 249, 7140 (1974).
13. G. Litwack, B. Ketterer and I. Arias, Nature, Lond. 234, 466 (1971).
14. W. Habig, M. Pabst, G. Fleischner, I. Arias and W. Jakoby, Proc. nat. Acad. Sci.U.S.A. 71, 3879 (1974).
15. N. Kaplowitz, G. Clifton, J. Kuhlenkamp and J. Wallin, Biochem. J., in press.
16. M. Dixon, Biochem. J. 55, 170 (1953).
17. H. Lineweaver and D. Burk, J. Am. chem. Soc. 56, 568 (1934).
18. N. Kaplowitz, J. Kuhlenkamp and G. Clifton, Biochem. J. 150, 259 (1975).
19. G. Clifton, N. Kaplowitz, J. Wallin and J. Kuhlenkamp, Biochem. J. 150, 259 (1975).
20. G. Clifton and N. Kaplowitz, Clin. Res. 24, 104A (1976).