Studies on the mechanism of action of 6-mercaptopurine

Interaction with copper and xanthine oxidase

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Interaction between 6-mercaptopurine, Cu^{2+} and the enzyme xanthine oxidase (EC 1.2.3.2) was examined. Whereas Cu^{2+} was found to inhibit the enzyme, 6-mercaptopurine could protect as well as reverse the enzyme inhibition produced by the metal ion. The formation of a complex between 6-mercaptopurine and Cu^{2+} seems to be responsible for the observed effect. Job's [(1928) *Ann. Chem.* **9**, 113] method has shown the composition of the complex to be 1:1. The apparent stability constant (log *K* value), as determined by Subhrama Rao & Raghav Rao's [(1955) *J. Sci. Chem. Ind. Res.* **143**, 278], method is found to be 6.74. It is suggested that the formation of a stable complex between 6-mercaptopurine molecules and Cu^{2+} may be an additional mechanism of action of 6-mercaptopurine, particularly with reference to its anti-inflammatory properties.

Besides the major use of 6-mercaptopurine as an anti-neoplastic agent (Calabresi & Parks, 1975), it has found additional application as an immunosuppressive (Hitchings & Elion, 1963) and anti-inflammatory drug (Page *et al.*, 1962). Although the mechanism of its anti-neoplastic action (interfering with nucleic acid biosynthesis; Calabresi & Parks, 1975) and of immunosuppression (inhibiting antibody production; Hitchings & Elion, 1963) is well documented, the biochemical basis of its anti-inflammatory activity in diseases such as hepatitis (Page *et al.*, 1962), collagen diseases (Lorenzen & Videbaek, 1965), ulcerative colitis (Bean, 1966) etc. is not very well explained by the described mechanisms of action.

One of the mechanisms underlying the anti-inflammatory action of various drugs has in recent years been explained by the formation of their copper chelates (Elmes, 1974). However, no such information regarding the anti-inflammatory activity of 6-mercaptopurine being mediated through the formation of its copper chelate is available. Tissue and serum copper concentrations are known to be elevated in malignancy, as well as in inflammatory processes, and are an extremely sensitive indicator of these conditions (Hrgovcic *et al.*, 1973). Moreover, a relationship between copper concentration and activity of the disease and the effectiveness of

the therapy exists. High copper concentrations are observed in active disease, diminishing with the abatement of the disease in response to the therapy (Hrgovcic *et al.*, 1973; Fleming *et al.*, 1974). Furthermore it is also reported that the activity of xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2), the enzyme responsible for the metabolism of 6-mercaptopurine, is decreased in neoplastic disorders (Prajda *et al.*, 1976). The altered activity of this enzyme appears to be characteristic of neoplastic transformations (Weber, 1973). Cu^{2+} , in addition, is known to be a potent inhibitor of xanthine oxidase (Bergel & Bray, 1959).

These facts indicate the possibility that an interrelationship between the action of 6-mercaptopurine, Cu^{2+} and xanthine oxidase could exist under the conditions cited above. In the present study, therefore, we have tried to examine the interaction between them, since no such information is available.

Materials and methods

Chemicals

Xanthine, 6-mercaptopurine and calcium phosphate gel were obtained from Sigma. 2,3,5-Triphenyltetrazolium chloride and CuSO_4 were purchased from B.D.H. All other chemicals were of analytical grade.

Preparation of xanthine oxidase

Milk xanthine oxidase was prepared by the

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method of Massey *et al.* (1969). The final purification of the enzyme on Ca_3PO_4 gel was done by the 'Alternate step 3' (Massey *et al.*, 1969). A unit of enzyme activity is defined as $1\mu\text{mol}$ of xanthine oxidized/min, as described by the above authors. Protein was determined by the method of Lowry *et al.* (1951).

Enzyme assay

Enzyme activity was measured by the colorimetric method of Owen (1971), with the following modifications: The final assay conditions were adjusted to maintain the enzyme activity linear with respect to time for more than 5 min. The assay mixture, in a final volume of 5 ml, contained, in a Thunberg tube: 1 ml of phosphate buffer, pH 7.4, $500\mu\text{mol}$; 2,3,5-triphenyltetrazolium chloride, $5\mu\text{mol}$; 0.05 ml of the diluted enzyme and different concentrations of 6-mercaptapurine and Cu^{2+} as indicated. Into the lid was placed xanthine ($1\mu\text{mol}$). The tubes were evacuated, filled with N_2 , and the contents of the tube and the lid were mixed to initiate the reaction. Acetic acid was added to terminate the reaction after 5 min incubation at 37°C . The formazan produced was extracted with toluene and the A_{495} measured. A ΔA_{495} of 0.1/min corresponded to 1 unit of enzyme activity.

Absorption spectra

The absorption spectra of 6-mercaptapurine and 6-mercaptapurine- Cu^{2+} complex were recorded with a Beckman spectrophotometer, model DU-2400. All cuvettes used had a 1 cm light-path.

Stoichiometry

Job's (1928) method of continuous variation with equimolar solutions of the reagents was used to determine the stoichiometry of the complex. A number of series of solutions were prepared, each with a different concentration of Cu^{2+} and 6-mercaptapurine. Since the maximum difference in the absorbance between 6-mercaptapurine and 6-mercaptapurine- Cu^{2+} complex was observed at 318 nm, this wavelength was used to record the absorbances of the solutions. Absorbance was also recorded for the same number of 6-mercaptapurine solutions, used as blanks, which were prepared under identical conditions except for the addition of the metal. The difference in the absorbance of each of the above solutions and the corresponding blank was plotted against the molar fraction of copper to obtain 'Job's (1928) curves'.

Stability constant

The stability constant (K) was calculated from Job's (1928) curves by the method of Subhrama Rao & Raghav Rao (1955), by employing the formula:

$$K = (1 - \alpha)/\alpha^2 C$$

where C is the total concentration of the complex in

nmol/litre and α is the degree of dissociation was calculated by the formula:

$$A_m - A_s/A_m$$

where A_m is the maximum absorbance obtained from the horizontal portion of the curve, indicating that all reagent is present in the form of chelate. A_s is the absorbance at the stoichiometric molar ratio of the complex, the total concentration of the complex being equal to the concentration of the reagent.

Results and discussion

Incubation of xanthine oxidase with various concentrations of 6-mercaptapurine produced a slight activation of the enzyme activity at low concentrations of the purine. Control experiments, performed simultaneously in the absence of xanthine, did not reveal any substrate activity of 6-mercaptapurine at these concentrations of the purine. Increasing the concentration of 6-mercaptapurine, however, resulted in the inhibition of the enzyme, as seen from Fig. 1.

Xanthine oxidase was markedly inactivated by Cu^{2+} (Fig. 1). A definite inhibition of the enzyme activity was observed with concentrations of Cu^{2+} as low as $5\mu\text{M}$, whereas maximal inhibition (96.7%)

Fig. 1. Concentration-dependence of the alteration of xanthine oxidase activity by 6-mercaptapurine and Cu^{2+}

Each point represents the final activity obtained in an incubation mixture under the conditions described in the Materials and methods section. ●, Cu^{2+} at the concentration shown on the lower scale; ▲, 6-mercaptapurine at the concentration shown on the upper scale.

was produced by 50 μM - Cu^{2+} . The Lineweaver-Burk (1934) plots in the absence and presence of Cu^{2+} revealed the inhibition to be non-competitive. The apparent K_i value calculated from these data was found to be $9 \times 10^{-6} \text{ M}$.

Since in our previous work with pyruvate kinase (Vijayvargiya *et al.*, 1970) it was observed that D-penicillamine, a thiol compound with known anti-inflammatory activity, could protect against as well as reverse the inhibition of the enzyme by Cu^{2+} , we tried to examine whether such a relationship holds good or not with 6-mercaptopurine and Cu^{2+} -inhibited xanthine oxidase. Table 1 demonstrates that the addition of 6-mercaptopurine (20 μM) after the addition of low concentrations of Cu^{2+} (5 μM) led to complete protection of the enzyme against Cu^{2+} inhibition. 6-Mercaptopurine, however also provided protection of the enzyme against the inhibition produced by higher concentrations of this cation.

The possibility that 6-mercaptopurine treatment could also effect the re-activation of Cu^{2+} -inactivated xanthine oxidase was further investigated. The results (Fig. 2) demonstrate that preincubation of the enzyme for 10 min with 2.5–10 μM - Cu^{2+} markedly decreased the activity of xanthine oxidase. When 6-mercaptopurine was added to the incubation mixture, the inhibition observed with the lower concentrations of Cu^{2+} was reversed. 6-Mercaptopurine, however, failed to produce complete reversal of the enzyme inhibition at higher concentrations of Cu^{2+} . The data thus indicate the possibility of irreversible inactivation of the enzyme by copper.

During the course of the above experiments it was observed that, on the addition of 6-mercaptopurine to the reaction mixture containing Cu^{2+} , a spontaneous change from colourless to a yellow colour occurred. This indicated the possibility of complex-formation between 6-mercaptopurine molecules and Cu^{2+} ions. In order to confirm the formation of the complex, absorption spectra of 6-mercaptopurine and 6-mercaptopurine- Cu^{2+} com-

plex were compared by recording their absorbance at pH 8.0 between 250 and 350 nm. A characteristic alteration in the absorption spectrum of 6-mercaptopurine was observed when equimolar solutions of 6-mercaptopurine and Cu^{2+} (as CuSO_4) were mixed. The maximum absorption by 6-mercaptopurine was observed at 318 nm, whereas no such peak could be detected for the complex. Moreover, the absorption by 6-mercaptopurine was much more than that by the 6-mercaptopurine- Cu^{2+} complex, the maximum difference being at 318 nm.

Fig. 2. Reversal by 6-mercaptopurine of xanthine oxidase inhibition by Cu^{2+}
A sample (0.4 ml) of purified diluted enzyme was preincubated on ice for 10 min with 0.4 ml of Cu^{2+} (2.5–10 μM). A 0.2 ml portion was withdrawn from the preincubation mixture and mixed with 0.2 ml of either water or 6-mercaptopurine (20 μM), and this was then used for assaying xanthine oxidase activity. ●, Without 6-mercaptopurine treatment; ▲, 6-mercaptopurine-treated enzyme.

Table 1. Protective action of 6-mercaptopurine against Cu^{2+} inhibition of xanthine oxidase
 Cu^{2+} (at three different concentrations) was added to the reaction mixture just before the addition of 6-mercaptopurine (20 μM). Each value is the average for three separate experiments.

Addition	Concn. (μM)	Xanthine oxidase activity (% of control)
None (Control)		100.0
6-Mercaptopurine	20	114.0
Cu^{2+}	5	64.1
	10	47.0
	20	22.7
Cu^{2+} + 6-mercaptopurine	5 + 20	103.0
	10 + 20	84.2
	20 + 20	82.6

Fig. 3. Job's (1928) curves (equimolar) Concentrations of the reagents: A, [6-mercaptopurine] = [Cu²⁺] = 20 μM; B, [6-mercaptopurine] = [Cu²⁺] = 16.6 μM. The values are averages for six separate experiments.

The stoichiometry of the complex, as determined by Job's (1928) method of continuous variation with equimolar solutions of the reagents is presented in Fig. 3. It is evident from these curves that Cu²⁺ forms a 1:1 complex with 6-mercaptopurine. The apparent stability constant (log *K*), as calculated by Subhrama Rao & Raghav Rao's (1955) method is found to be 6.74 (S.E.M. ± 0.04).

The effect of the 1:1 6-mercaptopurine-Cu²⁺ complex on the enzyme activity was then studied (Table 2). Different concentrations of the complex did not produce as large a decrease in the xanthine oxidase activity as did similar concentrations of free Cu²⁺. The extent of inhibition was similar to that caused by 6-mercaptopurine alone (Fig. 1).

Our results have revealed Cu²⁺ to be an inhibitor as well as an inactivator of xanthine oxidase. In addition it exhibited the ability of forming a complex with 6-mercaptopurine. The mechanism by which 6-mercaptopurine protects and reverses copper inhibition of the enzyme activity thus becomes explainable by the process of chelation of Cu²⁺ ions by the drug. The physiological significance of the inhibition of xanthine oxidase by copper could be interpreted in terms of the regulation of the enzyme activity by the metal. It can further be suggested that

Table 2. Effect of Cu²⁺-6-mercaptopurine complex (1:1) on xanthine oxidase activity

Different concentrations of 6-mercaptopurine and Cu²⁺ solutions were mixed in 1:1 proportion and placed in the incubation mixture before the addition of xanthine.

[Cu ²⁺ -6-mercaptopurine] (mM)	Xanthine oxidase activity (% of control)
0.01	94.0
0.02	93.1
0.04	87.2
0.06	83.5

the reported decreased xanthine oxidase activity in neoplastic cells (Prajda *et al.*, 1976; Weber, 1973) may be related with the inactivation of the enzyme by the element.

Since formation of the copper complex of anti-inflammatory drugs has been shown to be associated with the mechanism of their action (Elmes, 1974), a similar process in the anti-inflammatory action of 6-mercaptopurine can be considered. Moreover, in addition to the described anti-metabolite action of 6-mercaptopurine in neoplastic disorders, it might also aid in the treatment of the disease by chelating copper, as a decrease in the concentration of copper is reported to be an index of the chemotherapeutic benefit of the degree of malignancy (Hrgovcic *et al.*, 1968). Furthermore, the stability constant of the copper-6-mercaptopurine complex is found to exceed those of several naturally occurring cellular chelators for copper (Bjerrum *et al.*, 1957; Foye, 1961). The magnitude of the stability constant thus provides a satisfactory indication for the proposed mechanism of action of 6-mercaptopurine as a drug. In conclusion, we believe that the above process can be an additional mechanism of action of 6-mercaptopurine hitherto not described.

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