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STUDIES ON THE MECHANISM OF FORMATION OF 5-MERCAPTO-1-METHYL-4-NITROIMIDAZOLE, A METABOLITE OF THE IMMUNOSUPPRESSIVE DRUG AZATHIOPRINE*

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Abstract The imidazole derivatives listed below were compared in their ability to generate 5-mercapto-1-methyl-4-nitroimidazole *in vivo*; this latter metabolite is formed during immunosuppressive therapy with azathioprine. The compounds tested were: 5-chloro-1-methyl-4-nitroimidazole, 5-glutathionyl- and 5-cysteinyl-1-methyl-4-nitroimidazole. These studies, carried out in mice and dogs, suggested that mercaptoimidazole is formed *in vivo* from the glutathione-imidazole adduct. Further investigations on urinary thioimidazole excretion in patients and in dogs treated orally and intravenously with azathioprine have indicated that azathioprine is very likely absorbed *per se* from the gut and not the stomach.

5-MERCAPTO-1-methyl-4-nitroimidazole (mercaptoimidazole) has been identified as a bright orange–yellow compound in the urine of patients undergoing immunosuppression with azathioprine.[1,2] Mercaptoimidazole is also formed *in vitro* by the reaction of azathioprine with glutathione under strongly alkaline conditions.[1] Since the thiolysis of azathioprine to 6-mercaptopurine involves a cleavage at the sulphur-imidazole bond,[2] it is obvious that the sulphur atom present in mercaptoimidazole must be derived from some other source. Because glutathione is the most abundant sulphydryl compound in mammals,[3] it is likely that most of the thiolysis of azathioprine *in vivo* will occur by reaction with glutathione, resulting in the production of 6-mercaptopurine and 5-glutathionyl-1-methyl-4-nitroimidazole (GSIM).

In this paper, the ability of the glutathione-imidazole adduct (GSIM) to generate mercaptoimidazole *in vivo* and *in vitro* was studied and compared in this conversion with the corresponding cysteinyl adduct (5-cysteinyl-1-methyl-4-nitroimidazole, CSIM). In addition, an attempt has been made to identify and estimate thioimidazoles in the urine of animals (including man) receiving azathioprine and other potential thioimidazole precursors.

MATERIALS AND METHODS

Synthesis of 5-glutathionyl- and 5-cysteinyl-1-methyl-4-nitroimidazole. A solution of azathioprine (50 mg) in 100 ml 2% NH_4CO_3 was mixed with 100 mg reduced glutathione. The ratio of the extinction at 320 nm (λ_{max} 6-mercaptopurine) to that at 280 nm (λ_{max} azathioprine) increased after the addition of glutathione and was measured

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at 30-min intervals; after 1 hr the ratio was constant (3·0), indicating the complete conversion of azathioprine to 6-mercaptopurine and GSIM. The glutathione-imidazole adduct was separated from other reaction products by descending chromatography on Whatman 3 MM paper using water as the mobile solvent (R_f GSIM, 0·92; R_f 6-mercaptopurine, 0·67). An additional yellow band was located at R_f 0·83. The GSIM band was located by its purple fluorescence under u.v. light (254 nm); after elution into water and evaporation of the water extract under vacuum, the compound was finally isolated as a light yellow-coloured oil. The yield of GSIM, based on the quantity of mercaptoimidazole generated from it (see below), was 42 per cent after chromatographic separation.

The cysteinyl-imidazole adduct (CSIM) was prepared by the same procedure except that 50 mg cysteine hydrochloride monohydrate was used in place of glutathione (R_f CSIM, 0·87).

Both thioimidazoles were soluble in water and ethanol and insoluble in petroleum ether, chloroform, ethyl acetate and benzene. Attempts to purify these compounds for analysis by crystalization and vacuum distillation resulted in extensive decomposition.

Synthesis of 5-chloro-1-methyl-4-nitroimidazole. 5-Chloro-1-methyl-4-nitroimidazole was synthesized by published methods from commercially available products (oxalic acid, methylamine and phosphorous pentachloride).[4–6]

Estimation of thiomidazoles in urine. Mercaptoimidazole was identified and estimated as previously described.[1] A urinary metabolite with similar behaviour to 5-glutathionyl-1-methyl-4-nitroimidazole in the presence of alkali ("GSIM"; see Fig. 1) was estimated in urine by determining the mercaptoimidazole generated from it under the conditions described below.

To 3 ml fresh urine was added 0·1 ml 5 N KOH. After 2 hr at room temperature, the solution was adjusted to pH 1–2 by the addition of 0·2 ml concentrated HCl. The decrease in the extinction, measured at 420 nm after the addition of 0·05 ml saturated mercuric chloride was adjusted for dilution ($\times$ 1·1) and used to calculate total urinary mercaptoimidazole, comprising mercaptoimidazole already present in the urine and that generated from "GSIM." Thus, the concentration of "GSIM" in the urine was measured from the difference in the mercaptoimidazole concentrations before and after alkali treatment, i.e. $[\text{"GSIM"}] = [\text{MIM}]_A - [\text{MIM}]_B$ where ["GSIM"] is the concentration of a GSIM-like metabolite in the urine; $[\text{MIM}]_A$ is the mercaptoimidazole concentration after alkali treatment; and $[\text{MIM}]_B$ is the mercaptoimidazole concentration before alkali treatment.

Further confirmatory evidence for the formation of mercaptoimidazole from "GSIM" was the maximal increase in extinction obtained at 420 nm under alkaline conditions (pH >10), in addition to the maximal decrease in extinction at 420 nm after acidifying with HCl and adding mercuric chloride as previously described.

Collection of urine samples. Mouse urine was collected onto two sheets of Whatman 3 MM paper layered at the bottom of the mouse cage; urine collections were made over a 2-hr period.

In the experiments with dogs, mongrel dogs weighing about 10 kg were used. Where time course studies were carried out, dogs were anesthetized intravenously with sodium pentobarbitone (30 mg/kg body weight) and the urine was collected using a catheter, half an hr before and hourly after the injection of the appropriate

substances; compounds were administered as solutions or as suspensions in isotonic saline (0·9%, w/v) for intravenous and intraperitoneal injections respectively.

RESULTS

Formation of mercaptoimidazole in vitro

The spectra of GSIM and CSIM, prepared and isolated as described, are shown in Fig, 1. Addition of alkali (to give 1 N NaOH) to GSIM resulted in λ_{max} at 420 nm and a shoulder at 280 nm with isobestic points at 366 nm and 294 nm; this product was identified as mercaptoimidazole by its u.v. and visible spectrum in the presence and absence of mercuric ions.[1] The peak extinction of CSIM at 380 nm decreased on the addition of alkali and an isobestic point at 322–325 nm was observed over the first hr after alkali addition.

Further confirmation for the chemical identities of GSIM and CSIM was obtained when these thioimidazoles were identified spectrophotometrically, both in neutral

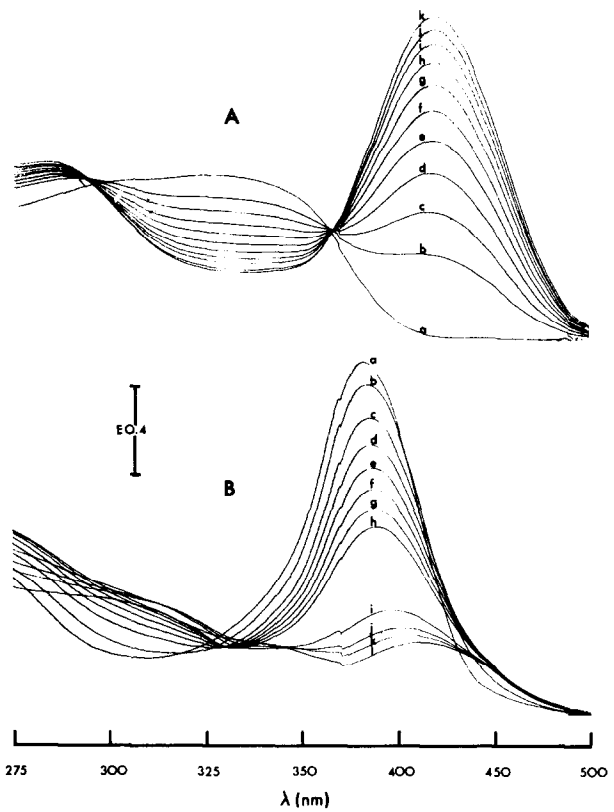


FIG. 1. Ultra violet and visible spectra of 5-glutathionyl-1-methyl-4-nitroimidazole (A, curve a) and the corresponding 5-cysteinyl derivative (B, curve a). Addition of sodium hydroxide to GSIM at a concentration of 1 N resulted in the formation of a compound with λ max, 420 nm; curve b was scanned 1 min after the addition of alkali and curves c to k at 10-min intervals after the addition (see A). Under the same conditions, CSIM gave a decrease in its peak absorbance at 380 nm. Curve b was scanned 1 min after alkali addition and curves c to h at 15-min intervals after the addition; curves i to l were scanned at 3·5, 4·5, 5·5 and 24 hr after the addition of alkali (see B).

and alkaline pH conditions, as the products of the reaction between chloroimidazole (100 μM) with glutathione and cysteine, respectively, in 0·05 M Tris buffered to pH 7·4.

The NMR spectra of GSIM and CSIM in D_2O were recorded and successive spectral scans made at 20-min intervals after the pH had been adjusted to 10 with potassium hydroxide (50 mg). To assist in the interpretation of these spectra, mercaptoimidazole, glutathione and cysteine were also scanned under identical conditions.

GSIM in D_2O yielded multiplets at δ 2·24, 2·58, 3·00 and 3·48 (all due to the glutathionyl group), one-proton singlets at 3·70, 1·99 and 7·88 (H atom the imidazole ring) and a three-proton singlet at 3·82 (*N*-methyl group of imidazole). At pH 10, there were multiplets at δ 1·94, 2·42, 2·96, 3·30 and 3·52 (due to the glutathionyl group), a three-proton singlet at 3·80 (*N*-methyl group of imidazole) and two singlets at 8·46 and 7·88 (aromatic protons, presumably due to hydrolysis of GSIM to two imidazole derivatives). Successive scans showed a new three-proton singlet at δ 3·54 (*N*-methyl group of mercaptoimidazole), a one-proton signal at 7·58 (H atom of mercaptoimidazole) and a two-proton doublet at 5·76 with a coupling constant of about 2 Hz (1,1-disubstituted vinyl group[7]). The peak at δ 7·88 decreased and multiplet peaks between 1·94 and 4·00 and a singlet at 8·46 were constant throughout successive scans in alkali.

The spectra of CSIM in D_2O yielded mutiplets at δ 3·79 (comprising the *N*-methyl of imidazole and the CH_2 group of cysteine), 3·42 (CH group of cysteine) and singlets at 7·2 and 7·7 (the two aromatic signals in this region may be due either to two imidazole derivatives or restricted rotation about the cysteinyl-S-imidazole bond). Addition of alkali resulted in an aromatic singlet at δ 7·32 (H atom imidazole), and multiplets at 3.50, 3·10 and 2·0 indicating extensive hydrolysis of the cysteinyl part of CSIM. At 20 min after the addition of alkali, there resulted a singlet at δ 8·44 and a broad multiplet peak from 2·5 to 4·2; these signals were unchanged in successive scans over an 80-min period.

Urinary excretion of thioimidazoles

Man. The excretion of mercaptoimidazole formed from azathioprine in man has already been described[1] and will not be dealt with in this paper except for the purposes of comparison. When mercaptoimidazole was given orally to two males (R. S., 0·5 mg/kg body weight, and A. C. 0·3 mg/kg body weight), the urinary excretion profile for mercaptoimidazole, shown in Fig. 2, was obtained. Peak excretion occurred between 4 and 5 hr compared with a maximum excretion of mercaptoimidazole at 2–3 hr when azathioprine (2 mg/kg) was taken orally by A. C. (Fig. 2). The total amount of mercaptoimidazole discharged into the urine in a 24-hr collection period represented 46 per cent of the original dose. By comparison, 7 per cent of an oral dose of azathioprine was excreted as mercaptoimidazole into the urine over the same period.

The amount of "GSIM" generated from azathioprine, estimated as described in the Materials and Methods section, was very low (0·6 per cent of the orally administered dose in 8 hr). It is likely that the amount measured may include some derived from azathioprine in the urine, since azathioprine added to control urines resulted in the formation of mercaptoimidazole (azathioprine added to control urine gave $\Delta m\epsilon$ at 420 nm with Hg^{2+} of 0·8). An attempt to overcome this interference by react-

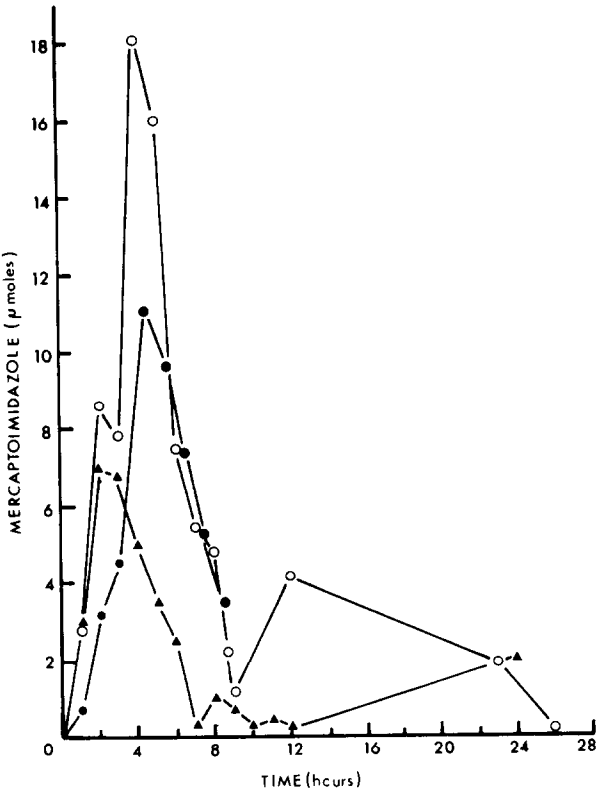


FIG. 2. Urinary excretions of 5-mercapto-1-methyl-4-nitroimidazole of two healthy males, R. S. (—○—) and A. C. (—●—), after an oral dose of this compound was taken at time O. For comparison, the urinary output of mercaptoimidazole, derived from an oral dose of azathioprine, is shown for A. C. (—▲—)

ing azathioprine with cysteine proved unsuccessful, as under these conditions GSIM lost the ability to generate mercaptoimidazole.

In a kidney transplant patient (E. L.), who 2 yr previously had received a total gastrectomy, the urinary excretion profiles for thioimidazoles, shown in Fig. 3, were obtained. The pattern of excretion of urinary mercaptoimidazole for patient E. L. was similar to that obtained with normal healthy males (cf. Fig. 3 with Fig. 2), although the total urinary output for this patient was less (2·6 per cent of azathioprine excreted as mercaptoimidazole in 24 hr).

"GSIM" excretion in E. L. appeared higher than that observed for other patients and healthy volunteers (1·7 per cent in 24 hr), though the significance of this finding is not known. The azathioprine excretion for E. L. was low (0·6 per cent in 24 hr; A. H. Chalmers, unpublished observation) and, therefore, unlikely to interfere with the "GSIM" estimation.

Dogs. Azathioprine (51 mg) given i.v. to a 12-kg dog resulted in a peak urinary excretion of mercaptoimidazole after 2 hr with a 5 per cent output in 7 hr. "GSIM" discharge into the urine was greater (15·3 per cent in 7 hr) with a peak excretion between 1 and 2 hr and none detectable after 3 hr (Fig. 4). Azathioprine interference in this instance was minimized by using large dilutions (1/50) for measuring GSIM in

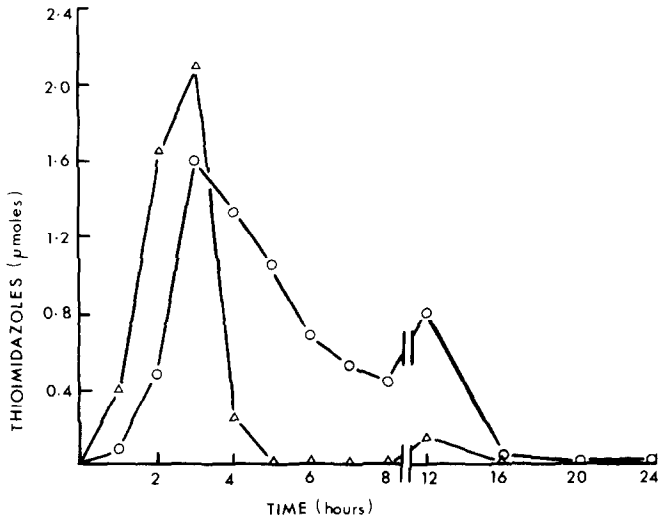


FIG. 3. Urinary excretion of mercaptoimidazole (–○–) and a 5-glutathionyl-l-methyl-4-nitroimidazole-type metabolite ("GSIM"–△–) of a kidney-transplanted, gastrectomized patient (E.L.) receiving oral azathioprine.

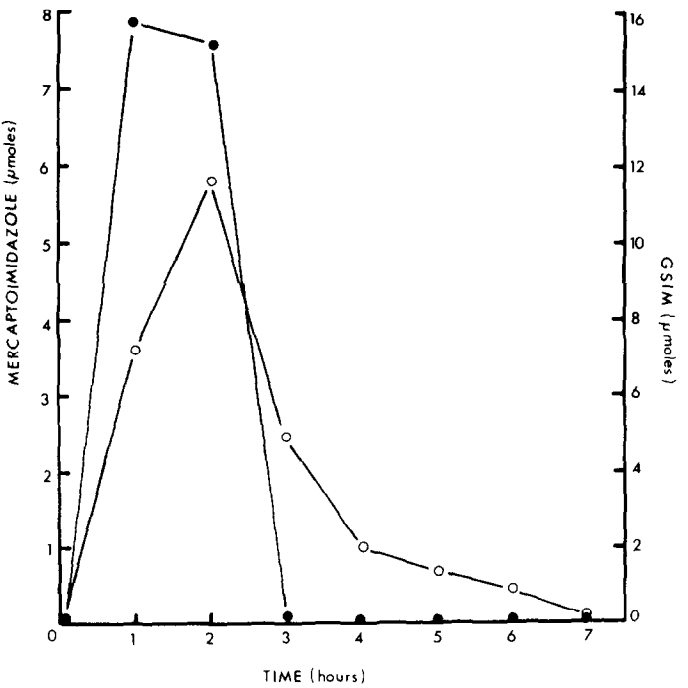


FIG. 4. Urinary excretion of mercaptoimidazole (–○–) and "GSIM" (–●–) after giving intravenously 51 mg azathioprine in isotonic saline to a 12-kg dog.

the assays. At these dilutions, the maximal concentration of azathioprine possible in the assay was 10 μM (in water, azathioprine reaches maximum solubility at a concentration of approximately 0·5 mM) and the contribution of azathioprine to GSIM estimation was small (less than 0·01 absorbance units). Oral administration of azathioprine (100 mg crushed in water) to two anesthetized dogs did not result in any detectable thioimidazoles excreted in the urine.

5-Chloro-1-methyl-4-nitroimidazole (chloroimidazole) was found to alkylate glutathione and cysteine *in vitro* at pH 7·4 with the resultant formation of GSIM and CSIM respectively. The ability of chloroimidazole to generate mercaptoimidazole and GSIM *in vivo* was, therefore, tested. Chloroimidazole (20 mg) given i.v. to a dog resulted in a low excretion of mercaptoimidazole in urine (0·5 per cent in 5 hr with a peak at 1 hr; Fig. 5A). A 60-mg dose given i.p. to a dog resulted in 3·7 and 0·8 per cent urinary excretions of mercaptoimidazole and "GSIM", respectively over a 5-hr collection period (Fig. 5B). The peaks for mercaptoimidazole excretion occurred at 2 hr and for "GSIM" excretion at 1–2 hr.

Mice. Chloroimidazole given i.p. at 150 mg/kg body weight to each of five mice resulted in 1·9 per cent of the original dose being excreted as mercaptoimidazole and 3·3 per cent as "GSIM" over a 2-hr period.

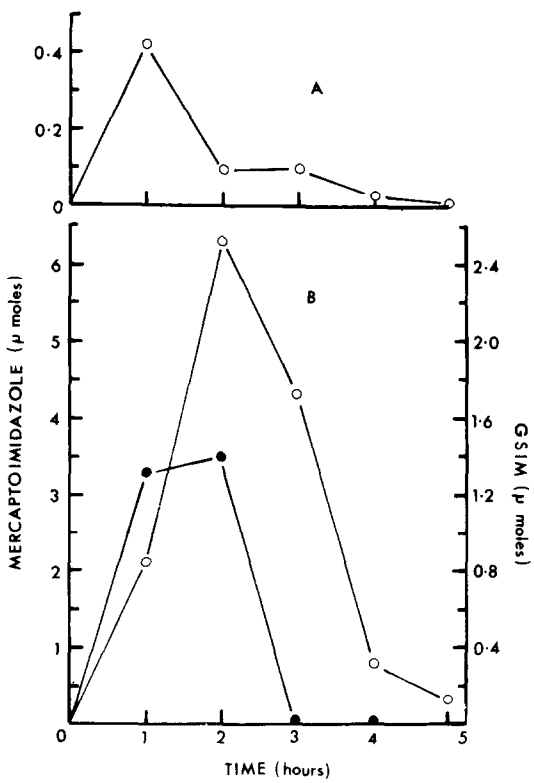


FIG. 5. (A) Urinary excretion of mercaptoimidazole after intravenous infusion of 20 mg 5-chloro-1-methyl-4-nitroimidazole in isotonic saline into the jugular vein of a 10-kg dog. (B) Urinary excretion of mercaptoimidazole (– ○ –) and "GSIM" (– ● –) after giving i.p. 60 mg 5-chloro-1-methyl-4-nitroimidazole in 100 ml isotonic saline to a 10-kg dog.

GSIM given i.p. to three mice (65 mg/kg) resulted in a discharge of 25 per cent mercaptoimidazole and 2·8 per cent of "GSIM" in a 2-hr collection period. In a corresponding experiment with CSIM, the yields of mercaptoimidazole and "GSIM" were much lower (0·05 and 0·01 per cent respectively).

The results of all experiments on urinary thioimidazole excretions have been summarized in Table 1.

TABLE 1. URINARY EXCRETION OF THIOIMIDAZOLE METABOLITES*

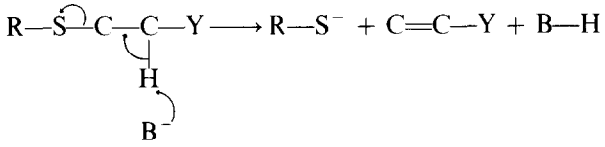
Species	Drug	Dose (mg/kg)	% Excretion mercaptoimidazole	"GSIM"
Man (A. C.)	Azathioprine	2·0, p.o.	7·0 (24)	0·6 (8)
Man (E. L.)	Azathioprine	1·5, p.o.	2·6 (24)	1·7 (24)
Man (A. C.)	Mercaptoimidazole	0·3, p.o.	34·2 (8·5)	
Man (R. S.)	Mercaptoimidazole	0·5, p.o.	46 (24)	
Dog	Azathioprine	4·3, i.v.	5·0 (7)	15·3 (7)
Dog	Azathioprine	10·0, p.o.	N.D.† (6)	N.D. (6)
Dog	Chloroimidazole	2·0, i.v.	0·5 (5)	N.D. (5)
Dog	Chloroimidazole	6·0, i.p.	3·7 (5)	0·8 (5)
Dog	Mercaptoimidazole	5·0, i.v.	46 (3)	
Mouse	Chloroimidazole	150, i.p.	1·9 (2)	3·3 (2)
Mouse	GSIM	65, i.p.	25·0 (2)	2·8 (2)
Mouse	CSIM	38, i.p.	0·05 (2)	0·01 (2)

* Excretion expressed as percentage of the dose given over the time course (hr) indicated in parentheses.

† N.D., not detected.

DISCUSSION

Unlike GSIM, CSIM failed to yield mercaptoimidazole under alkaline conditions. It is likely that this base-catalysed reaction follows the bi-molecular E_2-elimination mechanism of Hughes and Ingold,[8] described in a review by Tarbell and Harnish.[9]



Before it will occur, this elimination requires an electronegative Y group, resonance stabilization of both the RS^- and C=C—Y groups and strong base B^-. Glutathione satisfies most of these requirements and so the base-catalysed elimination for the formation of mercaptoimidazole from GSIM possibly occurs by the mechanism out-

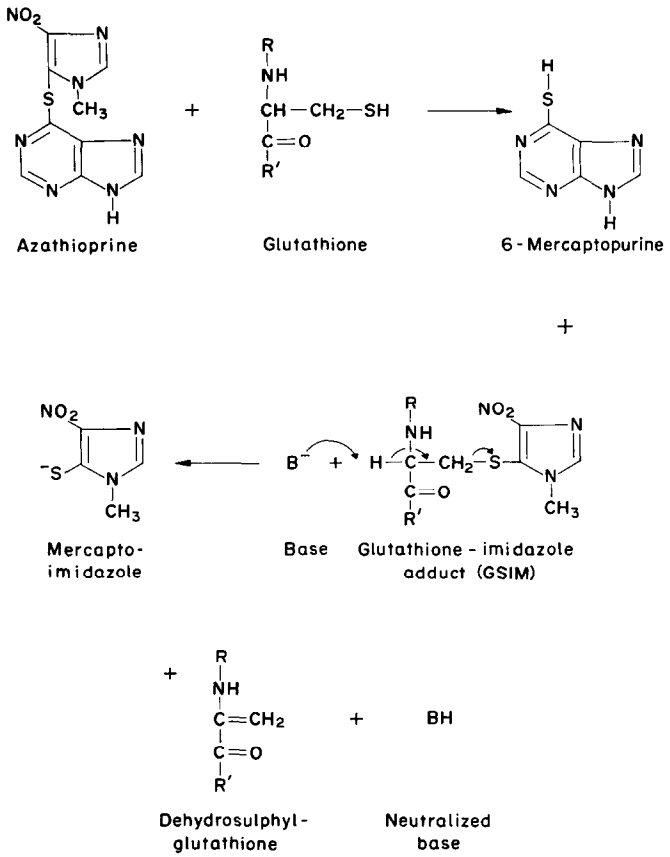


FIG. 6. Reaction sequences involved in the thiolysis of azathioprine by glutathione to 6-mercaptopurine and the formation of 5-mercapto-1-methyl-4-nitroimidazole from a base-catalysed elimination reaction on the corresponding 5-glutationyl-imidazole adduct. R = -L-glutamyl; R' = glycine.

lined in Fig. 6. The NMR spectrum of GSIM though complex, due possibly to some breakdown of GSIM after chromatography, does indicate a base-catalysed conversion to mercaptoimidazole (*N*-methyl, δ 3·54; imidazole hydrogen, δ 7·58) and the formation of an allyl group (δ 5·76) which is consistent with the formation of a dehydrosulphylglutathione like moiety. These NMR changes were not apparent with CSIM.

In these experiments, azathioprine administered orally to man resulted in a more rapid excretion of mercaptoimidazole than after administration of this thioimidazole itself. The following explanation may apply. (1) Azathioprine may be more rapidly absorbed than mercaptoimidazole from the gut and converted to mercaptoimidazole in the blood vascular system. (2) GSIM may be formed from azathioprine in the gut and taken up more rapidly than mercaptoimidazole into the blood. This is less likely, since glutathionyl substitution has been shown to increase the polarity and water solubility and decrease the lipid solubility of the parent compound thereby resulting in a lesser tendency of the glutathione substituent to penetrate cell barriers.[10]

The similarity in mercaptoimidazole excretion profiles of a gastrectomized transplant patient, E. L. to that of other transplant patients and healthy volunteers does support the concept that azathioprine is absorbed intact from the intestine into the plasma. Since the pH of the duodenum and jejunum may vary from 4 to 7,[11] the degree of conversion of azathioprine to 6-mercaptopurine in the gut will depend on the pH and sulphydryl concentration at this site of absorption (a pH in excess of 6·7 is required for the thiolysis of azathioprine[1]). If the absorption of azathioprine *per se* into the blood is important to its immunosuppressive activity, as it would appear,[12,13] then the thiolysis of azathioprine to 6-mercaptopurine, prior to adsorption, may be minimized by: (1) administering this drug to the patient directly after feeding, since the ingestion of food has been shown to lower the pH of the duodenum from about 7 to between 3·5 and 1·5 because of increased gastric emptying,[14] and (2) establishing diets for immunosuppressed patients which are low in sulphur content; feeding of cysteine and glutathione have been shown to increase the levels of free thiols in animal tissues.[15]

The experiments in which azathioprine was administered i.v. to a dog (Fig. 4) and chloroimidazole given i.p. to a dog (Fig. 5B) suggest that GSIM may be a precursor of mercaptoimidazole; GSIM peaks before mercaptoimidazole, and its over-all excretion time is more rapid. It is unlikely that azathioprine forms mercaptoimidazole by reaction with hydrogen sulphide in the colon because of the rapidity with which this thioimidazole is excreted.

The inability to detect thioimidazoles in the urine of dogs given oral azathioprine may be due to decreased peristalses in the anesthetized animals[16,17] and consequent movement of drug into the gut.

The specificity of the conversion of GSIM to mercaptoimidazole *in vivo* indicated the possibility of an enzyme-catalysed conversion perhaps similar to that described by Cantoni *et al.*[18] The enzyme isolated from the algae *Polysiphonia lanosa* was shown to catalyse the reaction below.

$$(CH_3)_2\text{–S–}CH_2\text{–}CH_2\text{–COOH} \longrightarrow (CH_3)_2S + CH_2\text{=CH–COOH}$$

Recent experiments have shown that GSIM and CSIM are both slowly converted to mercaptoimidazole at pH 7·4 in the presence of cell-free extracts of mouse liver; boiled extracts failed to effect this conversion. The rate of conversion of GSIM to mercaptoimidazole was 2·5–3·0 times that of CSIM (A. H. Chalmers, unpublished data).

It is generally believed that part of the immunosuppressive activity of azathioprine is inherent in its imidazole moiety.[19] We have recently shown that chloroimidazole significantly immunosuppressed the antibody response of mice to sheep red blood cells whereas GSIM was without effect.[20] It is likely, therefore, that the alkylating activity of the imidazole group, inherent in both chloroimidazole and azathioprine but not GSIM, may contribute to the immunosuppressive effects shown by these drugs.

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REFERENCES

1. A. H. CHALMERS, P. R. KNIGHT and M. R. ATKINSON, *Aust. J. exp. Biol. med. Sci.* **45,** 681 (1967).
2. G. B. ELION, *Fedn Proc.* **26,** 898 (1967).
3. G. E. GLOCK, in *Biochemists Handbook* (Ed. C. LONG), p. 793. E. & F. N. Spon, London (1961).
4. O. WALLACH, *Justus Liebigs Annln Chem.* **184,** 50 (1877).
5. F. F. BLICKE and H. C. GODT, *J. Am. chem. Soc.* **76,** 3653 (1954).
6. J. SARASIN and E. WEGMAN, *Helv. chim. Acta* **7,** 713 (1924).
7. D. W. MATHIESON, in *NMR for Organic Chemists*, p. 134, Academic Press, London (1967).
8. E. D. HUGHES and C. K. INGOLD, *Trans. Faraday Soc.* **37,** 657 (1941).
9. D. S. TARBELL and D. F. HARNISH, *Chem. Rev.* **49,** 1 (1951).
10. B. B. BRODIE, J. R. GILLETTE and B. N. LADU, *A. Rev. Biochem.* **27,** 427 (1958).
11. A. BENN and W. T. COOKE, *Scand. J. Gastroent*, **6,** 313 (1971).
12. G. B. ELION, S. CALLAHAN, S. BIEBER, G. H. HITCHINGS and R. W. RUNDLES, *Cancer Chemother. Rep.* **14,** 93 (1961).
13. BURROUGHS WELLCOME INC. *Information for Investigators*; 6-(1-*Methyl*-4-*nitro*-5-*imidazolyl*)-*thiopurine*, p. 5 (1963).
14. R. A. ROVELSTAD and F. T. MAHER, *Gastroenterology* **42,** 588 (1962).
15. C. R. BALL, *Biochem. Pharmac.* **15,** 809 (1966).
16. C. L. BURSTEIN, *Proc. Soc. exp. Biol. Med.* **40,** 122 (1939).
17. J. P. QUIGLEY, O. W. BARLOW and C. K. HIMMELSBACK, *J. Pharmac. exp. Ther.* **50,** 425 (1934).
18. G. L. CANTONI, D. G. ANDERSON and E. ROSENTHAL, *J. biol. Chem.* **222,** 171 (1956).
19. G. H. HITCHINGS, *Fedn Proc.* **26,** 958 (1967).
20. A. H. CHALMERS, T. BURDORF and A. W. MURRAY, *Biochem. Pharmac.* **21,** 2662 (1972).