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PROTECTIVE EFFECT OF THIOSULFATE AND METABOLIC THIOSULFATE PRECURSORS AGAINST TOXICITY OF NITROGEN MUSTARD (HN_2)

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Abstract—Pharmacokinetic aspects of the protection by thiosulfate against HN_2 toxicity have been studied in mice. Determinations of blood concentrations of HN_2 following subcutaneous injection of the compound demonstrated a rapid resorption of the latter followed by a slower elimination from the blood stream. The effect of time of pretreatment with thiosulfate on the protective effect was correlated to the time course of the blood concentrations of the compound. The kinetics of the reaction between HN_2 and thiosulfate were studied *in vitro* at pH 7·4 and 37° and the rate constants for the cyclization of HN_2 to its aziridinium ion and for the reaction between the latter and thiosulfate were determined. Chloride ions (0·15 M) were not found to retard the cyclization of HN_2 to its aziridinium ion under these conditions. The results of the *in vivo* and *in vitro* experiments are compatible with the current hypothesis of thiosulfate protection being confined to the extracellular space. Attempts were also made to find new antidotes against HN_2 among compounds, which could give rise to thiosulfate inside the cell. Presumptive thiosulfate precursors were evaluated with respect to their thiosulfate-forming capacity *in vivo*, their reactivity with the aziridinium ion of HN_2 *in vitro* and their protective effect against HN_2 in mice. The most efficient thiosulfate precursors were alaninethiosulfonate, mercaptopyruvate, propanedithiosulfonate, thiotaurine, and methanethiosulfonate. Only mercaptopyruvate showed a high reactivity with the HN_2–aziridinium ion and was also the best protective agent, although inferior to thiosulfate in these respects.

THE NITROGEN mustard *N*,*N*-bis-(2-chloroethyl)-*N*-methylamine (HN_2) is a radiomimetic alkylating compound, which is used clinically as an antitumor agent. The protection by thiosulfate against the system-toxic effects of HN_2 has been convincingly demonstrated in animal experiments,[1–4] and with various success in human beings[1,3,4] subjected to antitumor therapy. This antidote action of thiosulfate is attributed to its rapid reaction with free HN_2 present in tissues, whereas the "biochemical lesion", caused by the mustard through an alkylation of nucleic acids,[5] is not reversed. Furthermore, it has been proposed[2,4] that this inactivation of the mustard occurs exclusively in the extracellular space to which exogenous thiosulfate is confined.[16] We have now scrutinized this hypothesis by a pharmacokinetic study. The protective effect of thiosulfate was thus studied with respect to the time interval between the administration of the antidote and the mustard and the results were related to the blood levels of the antidote and mustard found at different times after administration and to the kinetics of the reaction between thiosulfate and HN_2. The latter is in fact a two-step reaction, the first step being an intramolecular cyclization of HN_2 to *N*-2-chloroethyl-

N-methylaziridinium ion (Az), which then reacts with thiosulfate in the second step. Each step was therefore separately studied *in vitro* under conditions approaching those to occur in body fluids.

As exogenous thiosulfate in contrast to HN_2[7] poorly penetrates the cell membrane, it cannot detoxify any HN_2 which has entered the cells. This poses an obvious limit to the antidote effect, as pointed out by Connors *et al.*[2] However, it may be possible to circumvent this permeability barrier by administration of a metabolic thiosulfate precursor. Certain sulfur compounds have been reported to behave as thiosulfate precursors *in vitro* and *in vivo*. Sörbo[8] thus demonstrated the facile formation of thiosulfate from β-mercaptopyruvate and sulfite in the presence of a rat liver homogenate and suggested that this reaction was the ultimate step of thiosulfate formation, previously known to occur in the mammalian body. Cavallini and Stirpe,[9] on the other hand, reported that cysteine-*S*-sulfonate* was converted to thiosulfate after injection into rats and proposed that thiosulfate formation in mammals occurred by this route. In a later communication[11] from the same group other sulfur compounds of possible metabolic origin (Fig. 1) were tested as thiosulfate precursors in the rat. Among the compounds studied alaninesulfinate was effective but even better were two thiosulfonates, alanine-3-thiosulfonate and thiotaurine.

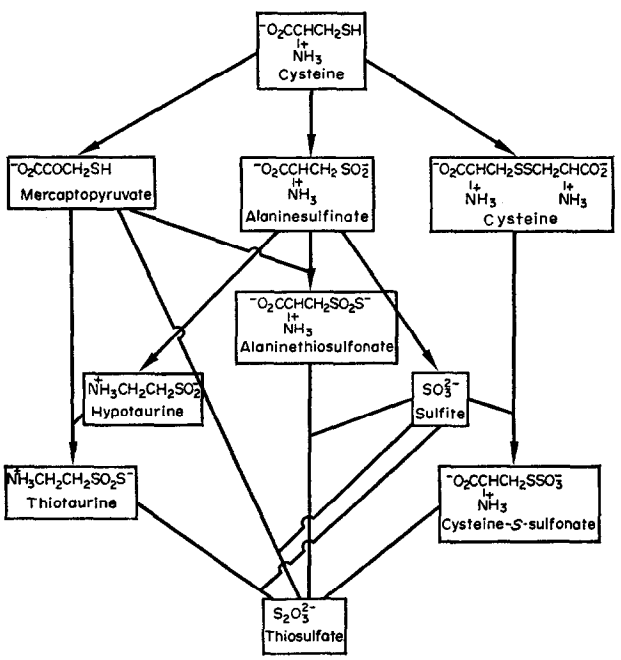

FIG. 1. Thiosulfate precursors and their metabolic relationship to thiosulfate and the amino acid cysteine.

In the second part of the present investigation the ability of the above mentioned thiosulfate precursors and some aliphatic thiosulfonates to protect mice against the system-toxic effects of HN_2 has been studied. The compounds investigated were also

* The nomenclature of sulfur oxy acids derived from cysteine used in the present paper is the "suffix system" of Savige and Maclaren[10].

compared with respect to their thiosulfate forming capacity in this animal as well as to their reactivity with Az *in vitro*.

MATERIALS AND METHODS

HN_2 (as the hydrochloride), prepared according to[12] was a gift of Mr. L. Fagerlind of this institute. Az was prepared *in situ* by preincubating a solution of HN_2 at pH 7·4 and 37° for 10 min. The sodium salts of methane$^-$, ethane$^-$ and propanethiosulfonic acid and of 1,3-propane-dithiosulfonic acid were synthesized from the corresponding sulfonyl chlorides according to the method of Troeger and Linde.[13] Thiotaurine,[14] sodium β-mercaptopyruvate[15] and sodium cysteine-*S*-sulfonate[16] were prepared according to the references indicated. Sodium alanine-3-thiosulfonate had to be prepared by an indirect route from the corresponding sulfinic acid, as attempts to convert the sodium salt of the latter to the thiosulfonate by established procedures always resulted in a mixture of the sulfinate and thiosulfonate. The synthesis finally developed was as follows. Alanine-3-sulfinic acid[17] was converted to the pyridinium salt of alanine-3-thiosulfonic acid by treatment with sulfur and pyridine according to[11] and an equimolar amount of sodium hydroxide was added to the product. The latter was taken to dryness on a rotary evaporator at 35°. The product was dissolved in a small amount of water and again taken to dryness and this step was repeated 4 times in order to remove any remaining pyridine. As attempts to obtain a crystalline preparation failed, the product was used without further purification after being assayed for cyanide-labile sulfur according to[8] and its purity had been established by paper chromatography.

Determination of HN_2 and thiosulfate in blood. Male CBA-mice of 18–24 g body wt were used in these experiments. HN_2 was given by the s.c. and thiosulfate by the i.p. route. Heparinized blood was obtained by puncture of the intraorbital plexus. Total HN_2 was determined in 0·30 ml blood after dilution to 1·0 ml with saline by the 4-(4′-nitrobenzyl)pyridine (NBP) method of Tan and Cole.[18] This method will include both NH_2 and Az in the determination. Thiosulfate was determined in 0·05 ml blood samples, deproteinized with tungstic acid, by a micromodification of a previously described colorimetric procedure.[19]

Determination of HN_2 and Az in kinetic experiments. The rate of cyclization of HN_2 to Az was assessed from colorimetric determinations of remaining HN_2 by an adaption of the NBP-method of Epstein *et al.*[20] As both HN_2 and Az react with NBP, HN_2 was separated from Az by extraction into benzene at an alkaline pH[12] and then re-extracted into acid before the colorimetric step. The determination was carried out as follows. To a 2 ml sample, containing up to 0·3 μmole of HN_2, was rapidly added 0·2 ml 0·1 M NaOH and 4 ml water-saturated benzene and extraction performed for 30 sec on a Vortex-mixer. (The reaction time was taken at the start of the benzene extraction.) A 3 ml aliquot of the benzene phase was then extracted with 3 ml of 0·01 M H_2SO_4 and 2 ml of the acid phase was adjusted to pH 4·6 with 0·2 M sodium acetate. The volume was made up to 4·0 ml with water, NBP was then added (0·4 ml of 5% in NBP in acetone) and the sample heated for 20 min in boiling water. After cooling in an ice-bath, 2 ml of triethylamine–acetone (1 : 1) were added and the absorbancy then determined exactly 90 sec later at 570 nm.

The reaction between Az and thiosulfate or thiosulfate precursors was followed colorimetrically. The method developed for this purpose was based on the ability of Az, being a quaternary nitrogen compound in contrast to the reaction products, to

form an ion-pair with dipicrylamine.[21] This ion-pair is coloured and can be extracted into an organic solvent. The analysis was carried out as follows. To a 4 ml sample, containing up to 0·08 μmole of Az, was added 1·0 ml 0·1 M NaOH and 5·0 ml ice-cold dipicrylamine reagent (10 mg dipicrylamine in 100 ml methylene chloride). The sample was vibrated for 10 sec on a Vortex mixer. (The end of the reaction time was taken at the beginning of the extraction step.) The organic phase was then filtered through a pledget of glass-wool, placed in a Pasteur-pipette, and the absorbancy at 420 nm was determined in a 1 cm cuvette. This method could also be used for determination of the cyclization rate of HN_2, and gave in fact results in satisfactory agreement with the method based on determination of remaining HN_2. The latter was preferred, as it was not affected by concomittantly occurring side reactions, such as hydrolysis of Az or reaction between Az and buffer components.

The sum of HN_2 and Az, remaining in a reaction system of HN_2 and thiosulfate, was determined by the NBP-method. However, the mustard entities must first be separated by ion-exchange chromatography from the sulfur compound, which otherwise would interfere in the colorimetric step. This method was also used for following the reaction between Az and β-mercaptopyruvate, as the latter interfered in the dipicrylamine assay. The analysis was carried out as follows. A 0·5 ml aliquot of reaction mixture, containing up to 0·25 μmole of HN_2 + Az, was rapidly applied to a small column (1·0 × 0·6 cm) of Dowex 50-X8 (100–200 mesh) in the hydrogen form. The end of the reaction time was taken when half of the sample had entered the column. The latter was immediately washed with water (5 ml) and the adsorbed mustard then eluted with 6 ml of 1 M KCl–0·1 M HCl. A 2·0 ml aliquot of the eluate was brought to pH 4·6 with sodium acetate and its volume was adjusted to 4·0 ml with water. The reaction with NBP and colour development was then carried out as previously described for the determination of uncyclized HN_2.

Thiosulfate formation in vivo *from sulfur compounds.* Male C 57 mice (18–30 g body wt), kept on a standardized diet, were placed in pairs in metabolic cages and the urine from each pair of animals was collected under toluene for 24-hr periods. After two control periods, the animals were injected i.p. with the compounds studied at the same dose level used in the protection experiments (vide infra). Urine was then collected during two following 24-hr periods. The urine samples were diluted to 20 ml with water, adjusted to pH 7·0 with 1 M NaOH and treated with one-tenth volume of 1 M cadmium acetate in order to remove interferring compounds.[8] The precipitate formed was removed by centrifugation and thiosulfate determined in an aliquot of the supernatant by the method of Sörbo.[19]

Protection studies. The animals used were male C 57 mice weighing 18–22 g. All compounds were given in saline solutions; HN_2 (as the hydrochloride) by s.c. injection in the nuchal region in a volume of 2·5 ml/kg body wt and the protective compounds by i.p. injection in a volume of 12·5 ml/kg. The dose of thiosulfate was 2·00 g/kg which was found to be well tolerated in these animals. Other protective compounds were given at a dose level equimolar with that of thiosulfate (8·06 mmoles/kg) after preliminary experiments on at least ten mice had shown that this dose was safely tolerated. In case of mercaptopyruvate this dose was too high but when reduced by the factor 0·7 (to 5·61 mmoles/kg or 1·00 g/kg) it was well tolerated. Mortality figures were determined in the protection studies 21 days after administration of the compounds and LD_{50} values were then calculated according to Weil[22] from results obtained with

4 dose levels of HN_2 and groups of four animals. The Dose Reduction Factor (DRF) for the protective compound was then calculated as the ratio (LD_{50} HN_2 with protective agent)/(LD_{50} HN_2 alone). The LD_{50} of HN_2 alone (as the hydrochloride) was 2·87 mg/kg (with 95 per cent confidence limits 2·55–3·19 mg/kg).

RESULTS

Blood levels of HN_2 and thiosulfate in relation to the protective effect of the latter. When the blood concentrations of the mustard and the protective agent were studied, the latter were administered by the same route as used in the protection experiments. In case of HN_2, the limited sensitivity of the analytical method used required that supralethal doses of the compound were given in order to obtain meaningful results. HN_2 was therefore studied at 3 dose levels in order to evaluate the relation between blood levels and the dose administered. As shown in Fig. 2 the blood concentrations had within 5 min reached peak values, which were proportional to the dose of HN_2. They then decreased with an average half-time of 15 min (range 13–20 min) as determined from semilogarithmic plots of the decreasing part of the curves (not shown).

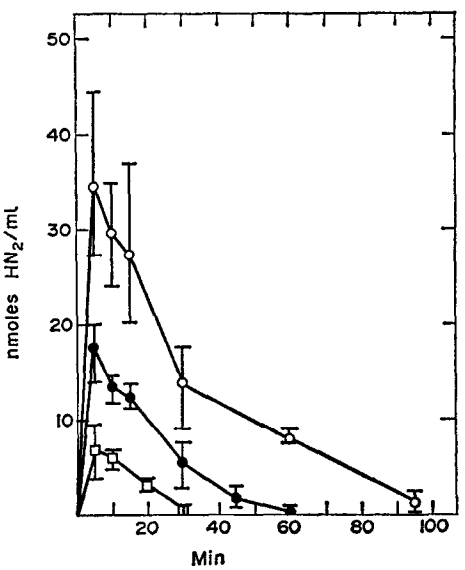

FIG. 2. Blood levels of HN_2 following s.c. injection of the compound. HN_2 injected at zero time. The vertical bars indicate the range from three determinations. Dose of HN_2 (as the hydrochloride): ○——○, 40 mg/kg; ●——●, 20 mg/kg; □——□, 10 mg/kg.

When thiosulfate was given, the blood levels reached a maximum 10 min after injection (Fig. 3) and then slowly decreased with a half-time of 38 min.

The protective effect of thiosulfate was then studied in relation to the time interval between administration of thiosulfate and HN_2. As shown in Fig. 4, no significant protection was observed when thiosulfate was given 10 min after HN_2, whereas the protective effect was maximal when the protector was given 10 min before HN_2 and then gradually decreased as the time of pretreatment was prolonged (half-time 29

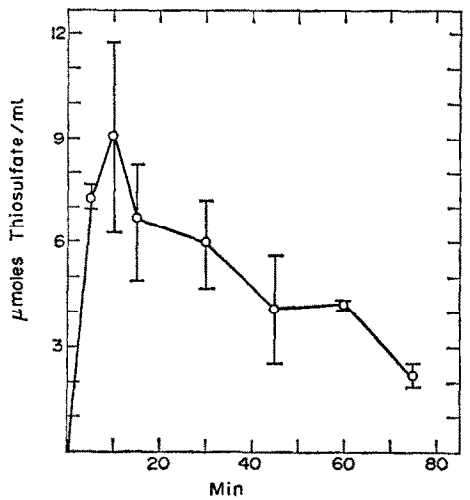

FIG. 3. Blood levels of thiosulfate following i.p. injection of the compound. Thiosulfate (2·00 g/kg) given at zero time. The vertical bars indicate range of measurements on three animals.

min). The protection effect thus appeared to follow the blood levels of thiosulfate, which would be expected if the rate of inactivation of HN_2 is proportional to the thiosulfate concentration, implying that the reaction is of the second order. However, this is not compatible with earlier reports in the literature[23–25] that the reaction between HN_2 and thiosulfate is of the first order and its rate thus independent of the thiosulfate concentration. This prompted us to re-examine the kinetics of the reaction between HN_2 and thiosulfate.

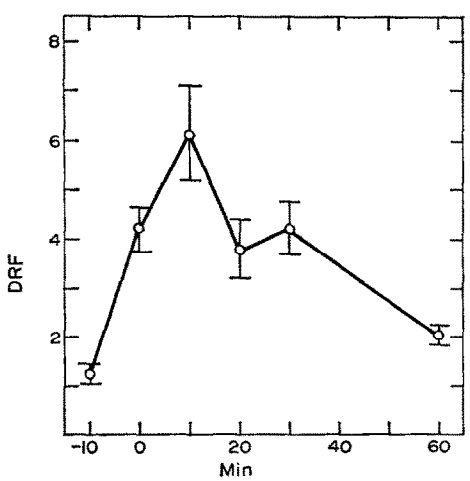

FIG. 4. Time dependence of protective effect of thiosulfate against HN_2. NH_2 was given at various times prior to (negative time values) or after (positive time values) thiosulfate. Vertical bars indicate the 95 per cent confidence interval.

Reaction between HN_2 and thiosulfate in vitro. Earlier studies[23,24] have demonstrated that the reaction between HN_2 and a nucleophilic agent, such as thiosulfate, follows the mechanism shown in Fig. 5. The initial step, which is of the first order, consists of a cyclization of HN_2 to Az. The latter is an alkylating agent, which may react with a vast number of nucleophiles; these reactions being of the second order. When the nucleophile is thiosulfate, a monothiosulfate ester (Bunte salt) is the initial product, which may react further with a second thiosulfate ion to give a dithiosulfate ester.[26] However, this secondary reaction is much slower[27] than the formation of the monothiosulfate ester and may be neglected in this context.

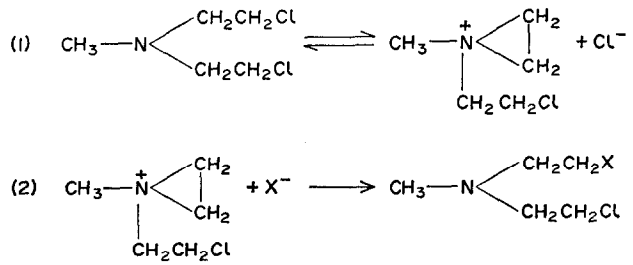

FIG. 5. Mechanism of reaction between HN_2 and nucleophilic agents. (1) Cyclization of HN_2 to Az, (2) Attack of nucleophile (X^-) on Az.

In order to elucidate the protective effect of thiosulfate against HN_2, the rate of conversion of HN_2 to Az and the rate of the reaction between the latter and thiosulfate should be determined under conditions approaching those to occur in body fluids. Thus the reactions were studied at pH 7·4, 37° and at 0·15 M chloride concentration. The latter is of special importance, as it has been reported[23] that chloride ions retard the formation of Az from HN_2 due to a reversion of the cyclization step (the "common

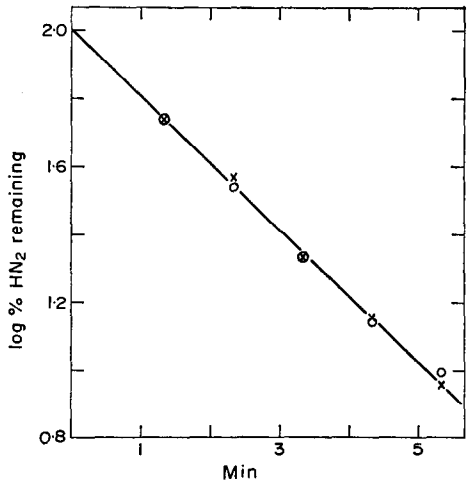

FIG. 6. Cyclization of HN_2 to Az. Reaction conditions: HN_2 1·5 × 10^{-4} M, NaCl or $NaNO_3$ 0·15 M, phosphate buffer 0·05 M, pH 7·4 at 37·2°. O——O, NaCl; ×——× $NaNO_3$.

ion effect"). When the rate of cyclization of HN_2 was measured under the conditions indicated above, the results (Fig. 6) demonstrate a rapid formation of Az with a first order velocity constant (k_1) of 0·45 min^{-1}. Chloride ions (0·15 M) did not significantly retard the reaction, as shown by replacing Cl^- with NO_3^- (Fig. 6). The reaction between thiosulfate and Az was then studied. Cohen *et al.*[28] assessed the rate of this reaction by an indirect approach, but direct measurements of the rate have not been reported previously, probably because of lack of a sensitive method for following the reaction. The requirement of a sensitive analytical method stems from the fact, that the reaction is of the second order with a high rate constant, and low concentrations of the reactants have to be used, in order to obtain not too fast reaction rates. The dipicrylamine method presented in this paper fulfils this requirement. Typical results from studies on the reaction between thiosulfate and Az are shown in Fig. 7. High initial thiosulfate–Az ratios were used in order to obtain pseudo first order conditions. As

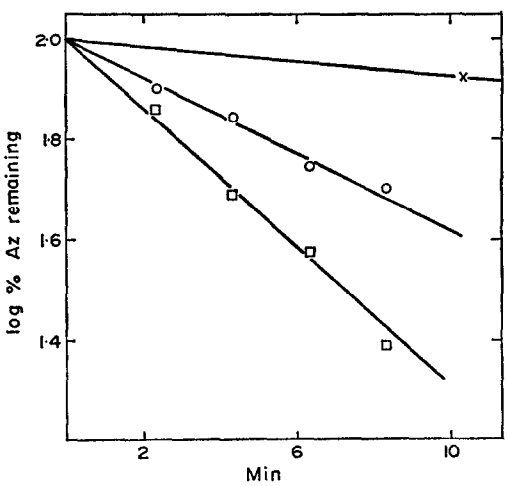

FIG. 7. Reaction between Az and thiosulfate. Reaction conditions (all concentrations refer to the final volume of the sample): HN_2 2 × 10^{-5} M, NaCl 0·15 M, phosphate buffer 0·05 M, pH 7·4. These components were preincubated for 10 min at 37·2° in order to convert HN_2 to Az. Thiosulfate was then added at zero time, and the reaction mixture analyzed for Az after various time intervals by the dipicrylamine method. □——□, Thiosulfate 8 × 10^{-4} M, ○——○, thiosulfate 4 × 10^{-4} M, ×, thiosulfate omitted.

shown in the figure, semilogarithmic plots of remaining Az vs time gave straight lines with slopes proportional to the thiosulfate concentration, in agreement with theory. The second order rate constant (k_2) was obtained as 181 $moles^{-1} \times 1 \times min^{-1}$.

However, not only the rate of disappearance of Az but also of HN_2 in the presence of thiosulfate should be considered. Only Az will react with thiosulfate, but the latter is confined to the extracellular space. Uncyclized HN_2 may therefore enter the mustard-sensitive cells and after intracellular cyclization to Az alkylate the "target molecules" without any competition from thiosulfate. The effect of thiosulfate on the sum of HN_2 and Az after various time of incubation with HN_2 was therefore studied (Fig. 8). It is evident that the rate of disappearance of the sum of the two toxic entities of the mustard is only to a limited extent dependent upon the thiosulfate concentration.

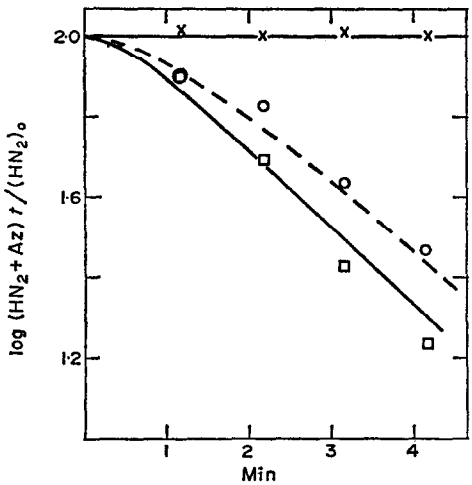

FIG. 8. Time course of disappearance of HN_2 and Az in the presence of thiosulfate. Reaction conditions: HN_2 5×10^{-4} M, NaCl 0·15 M, phosphate buffer 0·05 M, pH 7·4 at 37·2°. The sum of HN_2 and Az was determined by the NBP-reaction after ion-exchange chromatography. □——□, Thiosulfate 0·02 M; ○——○, thiosulfate 0·005 M. The lines were calculated according to equation (1), using a value 0·450 min^{-1} for k_1 and 181·1 × $moles^{-1} \times min^{-1}$ for k_2. ×, thiosulfate omitted.

Furthermore, as demonstrated by the semilogarithmic plot, the reactions is neither of the first, nor the second order. In fact, this system represents a chain of a true first order reaction (the cyclization step) followed by a pseudo-first order reaction between Az and thiosulfate. The order of the second step stems from the fact that the thiosulfate concentrations used were considerably higher than those of Az as they must be *in vivo*. It may be shown[29] that the following kinetic expression is valid in this case:

$$\frac{[HN_2]_t + [Az]_t}{[HN_2]_0} = \frac{k_1}{k_1 - k'_2} \times e^{-k'_2} \times t - \frac{k'_2}{k_1 - k'_2} \times e^{-k_1} \times t \qquad (1)$$

where $[HN_2]_0$ and $[HN_2]_t$ denote the concentrations of HN_2 at the start and at time t, respectively, $[Az]_t$ the concentration of Az at time t, k_1 is the first order rate constant for the formation of Az and k'_2 is the pseudo-first order constant for its disappearance in the presence of thiosulfate. Obviously, k'_2 is equal to the product of the second order rate constant (k_2) for the reaction between Az and thiosulfate and the concentration of the latter. When the previously determined values of k_1 and k_2 used to calculate the

$$\frac{[HN_2]_t + [Az]_t}{[HN_2]_0}$$

ratios according to equation (1), satisfactory agreement between the theoretical and the experimentally determined values was in fact obtained (Fig. 8). It should be noted that the higher thiosulfate concentration used in these experiments roughly correspond to that found in blood, when maximum protection against HN_2 is obtained (Figs. 3 and 4), and the lower concentration to that found 60 min after injection of thiosulfate, when also a much lower protective effect is observed. The fact that the rate of disappearance of the toxic entities of the mustard (HN_2 + Az) is only to a limited extent affected

by going from the higher to the lower thiosulfate concentration, in contrast to the protective effect, deserves some consideration and will be discussed later in this paper.

Formation of thiosulfate in vivo *from sulfur compounds.* When the presumptive thiosulfate precursors were evaluated with result to their ability to induce thiosulfate excretion in mice the results of Table 1 were obtained. Alanine thiosulfonate was the most active of the compounds studied, followed by mercaptopyruvate, propanedithiosulfonate, thiotaurine and methanethiosulfonate. Ethanethiosulfonate, propanethiosulfonate, cysteine-*S*-sulfonate and alanine sulfinate were less active although they all gave rise to significant amounts of thiosulfate.

TABLE 1. THIOSULFATE EXCRETION FOLLOWING ADMINISTRATION OF SULPHUR COMPOUNDS TO MICE*

Compound	Thiosulfate excretion (mmoles/kg body wt/24 hr)				Yield† (%)
	Before treatment		After treatment		
	Day 1	Day 2	Day 1	Day 2	
Thiosulfate	0·122 ± 0·028	0·107 ± 0·032	4·19 ± 1·12	0·180 ± 0·015	51·4
Mercaptopyruvate	0·114 ± 0·016	0·106 ± 0·011	0·764 ± 0·099	0·139 ± 0·001	12·2
Cysteine-*S*-sulfonate	0·139 ± 0·029	0·138 ± 0·031	0·458 ± 0·108	0·117 ± 0·019	3·7
Alaninesulfinate	0·112 ± 0·007	0·119 ± 0·009	0·287 ± 0·074	0·142 ± 0·014	2·5
Thiotaurine	0·115 ± 0·002	0·128 ± 0·002	0·828 ± 0·153	0·121 ± 0·013	8·8
Alaninethiosulfonate	0·143 ± 0·007	0·165 ± 0·009	1·47 ± 0·03	0·109 ± 0·016	15·8
Methanethiosulfonate	0·117 ± 0·004	0·130 ± 0·004	0·739 ± 0·108	0·159 ± 0·025	8·1
Ethanethiosulfonate	0·128 ± 0·018	0·122 ± 0·006	0·420 ± 0·029	0·164 ± 0·037	4·1
Propanethiosulfonate	0·093 ± 0·038	0·127 ± 0·024	0·427 ± 0·051	0·151 ± 0·015	4·4
Propanedithiosulfonate	0·136 ± 0·036	0·128 ± 0·017	0·929 ± 0·142	0·129 ± 0·021	9·9

* The excretion values are the mean ± S.E.M. from three cages, each containing two animals.

† The yield of thiosulfate relative to the compound administered is the difference between the sum of the excretion values after treatment and the sum of the values before treatment, divided with the dose of the compound administered. The latter was 8·06 mmoles/kg except in the case of mercaptopyruvate, where it for reasons outlined in the text was 5·61 mmoles/kg.

Interaction between Az and thiosulfate precursors in vitro. The spontaneous reactions between Az and the thiosulfate precursors were studied in order to obtain a necessary background for interpretation of their protective action against HN_2 *in vivo*. The experimental approach was, with one exception, similar to that used in case of thiosulfate; thus the disappearance of Az was followed with the dipicrylamine method and high thiosulfate precursor–Az ratios were used in order to obtain pseudo-first order kinetics. Typical results obtained with some representative thiosulfonates are shown in Fig. 9. In case of β-mercaptopyruvate (Fig. 10), however, this compound was found to interfere with the dipicrylamine method and we were forced to use the less sensitive and more cumbersome method for assay of Az, based on ion-exchange chromatography followed by colorimetric determination with NBP. From the results of these experiments the second-order rate constants for the thiosulfate precursors were calculated and are summarized in Table 2. It is evident that only mercaptopyruvate showed a reactivity of the same magnitude as that of thiosulfate, whereas other compounds studied had little or no reactivity.

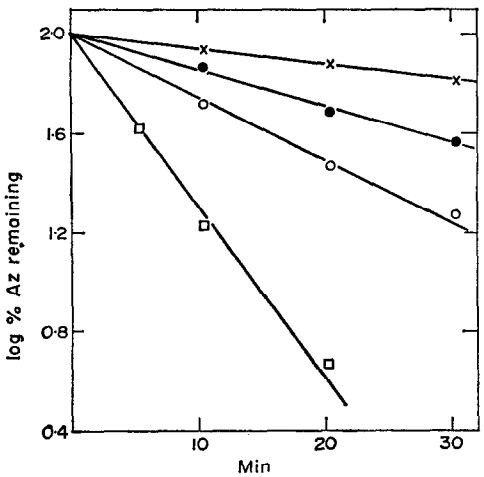

FIG. 9. Reactions between Az and thiosulfonates. Conditions as in Fig. 7, except that thiosulfate was replaced by the thiosulfonate at 0·02 M final concentration. □——□, Propanedithiosulfonate; ○——○, ethanethiosulfonate; ●——●, thiotaurine; ×——×, thiosulfonate omitted.

Protective effect of thiosulfate precursors. When thiosulfate and its precursors were given 10 min prior to HN_2 the protective effects shown in Table 3 were obtained. Among the precursors mercaptopyruvate gave the best protection, which, however, was inferior to that of thiosulfate. Cysteine-*S*-sulfonate, methanethiosulfonate, ethanethiosulfonate and propanedithiosulfonate gave a slight although significant protection, whereas other compounds were inactive. The protective effect of mercapto-

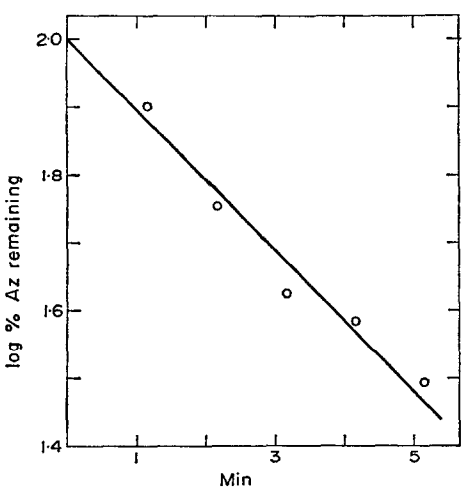

FIG. 10. Reaction between Az and β-mercaptopyruvate. Conditions as in Fig. 7, except that HN_2 was 5×10^{-4} M, β-mercaptopyruvate at 0·0025 M replaced thiosulfate and remaining Az was assayed by the NBP-reaction after ion-exchange chromatography.

TABLE 2. REACTION BETWEEN AZ AND SULPHUR COMPOUNDS*

Compound	k_2 / $1 \times mole^{-2} \times min^{-1}$
Thiosulfate	181
Mercaptopyruvate	95·8
Cysteine-*S*-sulfonate	0
Alaninesulfinate	1·09
Thiotaurine	0·95
Alaninethiosulfonate	2·29
Methanethiosulfonate	1·69
Ethanethiosulfonate	2·21
Propanethiosulfonate	2·18
Propanedithiosulfonate	7·22

* Reaction conditions as in Fig. 9 or in case of mercaptopyruvate as in Fig. 10. k_2 is the second order velocity constant, corrected for the disappearance of Az in buffer alone.

pyruvate, thiotaurine and alaninethiosulfonate, compounds very active as thiosulfate precursors, was also studied after prolonged periods of pretreatment (20 and 30 min; Table 4). The protective effect of mercaptopyruvate was not significantly augmented under these conditions, but thiotaurine and alaninethiosulfonate (which had no protective effect after 10 min time of pretreatment) gave in fact a significant protection after the prolonged times of pretreatment.

TABLE 3. PROTECTIVE EFFECT OF THIOSULFATE AND ITS PRECURSORS AGAINST HN_2*

Compound	DRF
Thiosulfate	6·07 (5·19–7·09)
Mercaptopyruvate	3·31 (2·56–4·39)
Cysteine-*S*-sulfonate	1·65 (1·38–1·96)
Alaninesulfinate	1·06 (0·94–1·18)
Thiotaurine	0·89 (0·40–2·00)
Alaninethiosulfonate	1·11 (0·49–2·52)
Methanethiosulfonate	1·50 (1·19–1·89)
Ethanethiosulfonate	1·25 (1·14–1·36)
Propanethiosulfonate	0·94 (0·85–1·04)
Propanedithiosulfonate	1·61 (1·23–2·12)

* The protective compounds were given 10 min before HN_2. DRF as defined in Materials and Methods. Values within brackets indicate the 95 per cent confidence interval.

TABLE 4. PROTECTION GIVEN BY THIOSULFATE PRECURSORS AFTER PROLONGED TIME OF PRETREATMENT

Compound	Time of pretreatment (min)	DRF*
Mercaptopyruvate	20	3·88 (3·08–4·89)
Mercaptopyruvate	30	3·10 (2·11–3·62)
Thiotaurine	20	1·50 (1·14–1·97)
Thiotaurine	30	1·94 (1·43–2·65)
Alaninethiosulfonate	20	1·45 (1·23–1·72)
Alaninethiosulfonate	30	2·27 (2·03–2·54)

* Values within brackets represent the 95 per cent confidence interval.

DISCUSSION

One major finding of the present investigation was that the protective effect of thiosulfate against HN_2 was correlated to the blood levels of thiosulfate. From the current hypothesis that the protective effect of thiosulfate is due to the compound acting as a "mustard scavenger" in the extracellular space, one should expect that the rate of reaction between HN_2 and thiosulfate should be proportional to the thiosulfate concentration. However, when this reaction was studied *in vitro* using thiosulfate concentrations corresponding to those found in the blood of protected animals, the disappearance of the two toxic entities of the mustard (HN_2 and Az) was only to a limited extent dependent upon the thiosulfate concentration. This is explained by the rate of the overall reaction between HN_2 and thiosulfate being under these conditions mainly determined by the rate of conversion of HN_2 to Az. The discrepancy between our *in vivo* and *in vitro* results may be reconciled if we assume that HN_2 to a large extent cyclisizes to Az during its resorption from the injection site, as the reaction between Az and thiosulfate is of the second order and its rate thus proportional to the thiosulfate concentration. This assumption is apparently justified, as judged from the value for the cyclization rate of HN_2 under physiological conditions, which corresponds to a half-time for uncyclized HN_2 of only 1·5 min. Moreover the biological half-time of total HN_2 in blood, measured by a method responding to both HN_2 and Az was about 10-fold higher than the half-time of HN_2, which signifies that the total HN_2 found in blood consists mainly of Az. The relatively slow disappearance of alkylating activity in blood after administration of HN_2 is noteworthy and was also recently observed by Williamson *et al.*[7] after intravenous injection of the mustard. Our pharmacokinetic data are thus compatible with (but do not prove) the current hypothesis of thiosulfate protection to HN_2 being due to a reaction between the protective agent and the mustard (or more precisely its aziridinium ion) in the extracellular space. The alternative hypothesis that thiosulfate enters mustard-sensitive cells to a certain extent and intracellularly competes with "target molecules" for Az cannot be excluded, but seems less attractive as the extracellular localization of exogenously administered thiosulfate appears to be well etablished.[6]

Furthermore, we found that chloride ions had no effect on the rate of conversion of HN_2 to Az. This is at variance to the results of Bartlett *et al.*,[23] who reported that

chloride ions had a strong retarding effect on this reaction due to the so-called "common-ion effect", implying a reversion of Az to HN_2 (Fig. 5). This prompted the suggestion[30] that HN_2 was prevented from conversion to Az in the extracellular space due to the high chloride concentration present in the latter. It was furthermore assumed that HN_2 entered cells much more rapidly than Az as cell membranes are in general relatively impermeable to ionic substances. However, as soon as HN_2 had penetrated the cell membranes, the much lower intracellular chloride concentration would not prevent the conversion of the mustard to its reactive product Az, which could then alkylate the "biological target molecules". This implies a significant role of chloride ions for the toxic action of HN_2. However, this interesting hypothesis is untenable in the light of the present work. The discrepancy between our results and those of Bartlett *et al.*[23] may be explained by the different experimental conditions used in the two investigations. We used conditions approaching those to occur in the extracellular fluid. Whereas Bartlett *et al.*[23] for technical reasons were forced to study the cyclization rate of HN_2 in acetone–water solutions. The effect of acetone in these experiments can in fact be predicted from the Hughes–Ingold theory[31] of solvent action to be a retardation of the cyclization of HN_2 and a promotion of the reverse reaction between Az and chloride ions. The latter may thus exert a retarding effect on the conversion of HN_2 to Az in acetone–water media, which does not appear in water solutions.

Another purpose of the present work was to find new antidoes against HN_2 among metabolic thiosulfate precursors, which in contrast to exogenously administered thiosulfate may result in an increased intracellular thiosulfate concentration. Various presumptive thiosulfate precursors were thus evaluated with respect to their ability to induce thiosulfate excretion in the mouse. Our results confirm and extend earlier findings[11] that thiosulfonates are metabolized to thiosulfate in the mammalian body. The mechanism behind is unknown, but it is plausible that rhodanese (thiosulfate: cyanide sulphurtransferase EC 2.8.1.1) participates in this conversion, as it has been demonstrated[32] that this enzyme catalyzes the reaction between a thiosulfonate and sulfite, with thiosulfate and the corresponding sulfinate as products. If thiosulfonates play a premordial role as thiosulfate precursors in the mammalian body as suggested by De Marco *et al.*[11] is on the other hand an open question. An alternative candidate is β-mercaptopyruvate, which previously[8] was shown to be converted to thiosulfate *in vitro*, and now has been found to behave in the same manner *in vivo*. However, the protection against HN_2 obtained with the thiosulfate precursors was in general much inferior to that of thiosulfate. It is possible that our goal of inducing increased intracellular concentrations of thiosulfate in the mustard-sensitive tissues (bone-marrow, spleen and lymphoid tissue) was not achieved due to a lack of the necessary enzymes in these tissues. The relatively high protective effect of mercaptopyruvate is nevertheless noteworthy. It is possible that this compound, in contrast to other thiosulfate precursors, was converted to thiosulfate in mustard-sensitive tissues. However, mercaptopyruvate also showed a high reactivity with Az and may owe its protective action to this effect. Unfortunately, mercaptopyruvate is more toxic and less protective than thiosulfate and thus offers no therapeutic advantages in comparison with this compound.

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