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PROTECTION AGAINST NITROGEN MUSTARD BY CYSTEINE AND RELATED SUBSTANCES, INVESTIGATED USING [^{3}H] METHYL-DI-(2-CHLOROETHYL) AMINE

S. F. CONTRACTOR*

Department of Biochemistry, St. Bartholomew's Hospital Medical College, Charterhouse Square, E.C.1.

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Abstract—Synthesis of [^{3}H] methyl-di-(2-chloroethyl) amine (HN2) is described. The protection afforded by cysteine and related compounds against the lethal action of HN2 was studied in mice. It was found, that in those mice protected by cysteamine and cystamine before the injection of the tritiated nitrogen mustard, the excretion of the radioactivity was greater than in those that were not thus protected. The possible mechanisms of this protective action are discussed in relation to the theories of chemical inactivation and formation of mixed disulphides with the SH group of the tissue proteins.

THE term "radiomimetic" has been coined to describe certain chemical compounds like the nitrogen mustards (e.g. methyl-di-(2-chloroethyl) amine, HN2) which display effects similar to those of ionizing radiation when applied to biological systems. Several parallelisms have been drawn between the two and the subject has been comprehensively reviewed by Philips.[1]

In 1951, the radioprotective action of cysteamine was first studied by Bacq[2] and other authors. This compound was found to give protection against lethal doses of X-irradiation to mice, if administered prior to the radiation. The logical consequence of this discovery was to see if the same protection was afforded by cysteamine against the effect of the "radiomimetic" agents. Peczenik[3] was amongst the first of the investigators to demonstrate that cysteamine, injected into rats with transplanted Walker carcinomas 30 min before the administration of nitrogen mustard, protected the rats against high doses of HN2. He further demonstrated that the pre-administration of cysteamine potentiated the therapeutic effect of lower doses of HN2, on rats suffering from the carcinoma. Thirkelsen[4] found that 80 per cent of C3H mice survived a minimum LD100 of HN2 when protected by injection of 150 mg of cysteamine per kg of mouse a few seconds to 30 min before the injection of HN2. If this time limit was changed to 60 min before or 10 min after the injection of HN2, the survival rate did not increase.

Several different compounds have been studied for their protective action against HN2 (Thirkelsen[5, 6], Brandt and Griffin,[7] Howe, Marvin and Spurr,[8] Mitchell and Girerd,[9] Goldenthal, Nadkarni and Smith[10]). Thirkelsen[5, 6] after studying a wide

* Present Address: Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, Goldhawk Road, London, W.6.

range of compounds came to the conclusion that for a compound to have protective action it must have a free —SH group.

The mechanism of this protective action is still not clear though different theories have been advanced to explain it. Foremost amongst these is the one based on chemical inactivation and put forward by Salerno and Friedell,[11] who showed that HN2 was inactivated when allowed to react with cysteamine or cysteine, and that the complex formed between the HN2 and either of the two compounds did not have a lethal effect when administered to animals. This theory would also explain the results obtained by Deysson and Truhaut,[12] who showed cysteamine to have a protective action against the effect of HN2 on certain plants grown in a medium containing a mixture of the two compounds. Thirkelsen[5] showed however, that the number of cells in the bone marrow of a protected animal was significantly higher than that of an unprotected animal and therefore concluded that a primary protection of the bone marrow was afforded by the cysteamine. The theory of chemical inactivation was also contested by Desaive and Varreto-Denoel,[13] who showed by means of histological and other findings, that a simple inactivation does not occur in the animal. The theory that anoxia is the mode of protection against radiation has been advanced by Patt[14] and this theory has also been proposed for protection against HN2. Kihlman[15] has shown, however, that the effect of HN2 is independent of the oxygen tension. Eldjarn and Pihl[16] advocated the theory that certain vital SH compounds in the cell form mixed disulphides with cysteamine and are thus protected against the effect of X-rays and HN2.

The present study was undertaken to investigate further the effect of cysteamine and less toxic related compounds, as protective agents against HN2. The elucidation of the protective mechanisms may hold the clue to the complex action of the HN2 itself.

MATERIALS

Synthesis of [^{3}H] *methyl-di-(2-chloroethyl) amine*

[^{3}H] *formic acid*

Anhydrous oxalic acid A.R. (11·0 g), T_2O (2 ml 0·4C), H_2O (2·4 ml) and glycerol (0·4 ml) were mixed with cooling. The mixture was then allowed to come to room temperature and gently warmed until evolution of CO_2 commenced, then placed in an oil bath at 180°–190° and refluxed for 16 hr. The reaction mixture was slowly distilled on an oil bath, giving formic acid as the distillate (7·2 ml). From a small sample of the distillate the concentration of the acid was determined by titration with alkali (13·28N) and hence the weight of formic acid (4·6 g) was calculated.

[^{3}H] *methyldiethanolamine*

[^{3}H] formic acid (4·6 g) in a round bottom flask (100 ml) was cooled in an ice bath. Diethanolamine (10·5 g) was slowly added followed by dropwise addition of 40% formaldehyde solution (8·0 ml). The reaction mixture was stirred continuously to avoid generation of excess heat and slowly allowed to come to room temperature. The reaction was now started by warming the mixture in a water bath until CO_2 was evolved. The mixture was removed from the water bath until evolution of CO_2 had ceased and then heated on the bath for 16 hr and distilled under reduced pressure on the water pump. The [^{3}H]-methyldiethanolamine was distilled under vacuum at 5 mm and 124° Yield = 10·55 g.

[^{3}H] *methyl-di-(2-chloroethyl) amine*

Purified $SOCl_2$ (23 g) in benzene A.R. (15 ml) was refluxed in a round bottom flask (100 ml). To the reflux mixture was added dropwise [^{3}H] methyldiethanolamine (10·55 g) in anhydrous benzene (15 ml) over a period of 1 hr. The mixture was then refluxed for 2 hr in an oil bath at 90–100°, cooled to room temperature, and water (15 ml) and ION–NaOH (15 ml) were added with cooling. The resultant oil was extracted into benzene, using three lots of 30 ml of benzene each. The combined benzene extracts were washed with water till neutral to litmus and dried over anhydrous sodium sulphate. After distillation of the benzene, the [^{3}H] methyl-di-(2-chloroethyl) amine was distilled under reduced pressure on an oil pump at a temperature low enough to avoid overheating (64°, 3 mm).

The distillate was dissolved in anhydrous benzene, cooled in an ice–salt bath, and the solution saturated with anhydrous HCl gas. The crystals of HN2—HCl were filtered off, yield = 11·8 g. The crystals were recrystallized once from acetone to give M.P. = 110°.

Radioassay of the crystals using the Tri-Carb Liquid scintillation spectrometer gave a count rate of 31,000 counts/min per mg in hyamine as detailed under counting procedures.

Hyamine (—OH *form*)

Hyamine-10 X from Rohm and Haas (100 g) was recrystallized from toluene which had been redistilled over calcium hydride.

The recrystallized hyamine (50·4 g), methanol A.R. (redistilled) (150 ml), and silver oxide (12·7 g) were shaken in a flask (250 ml) for ½ hr and centrifuged. The supernatant was exposed to a 100 W incandescent bulb for 72 hr and re-centrifuged. The concentration of the supernatant solution was found by titration with standard HCl to be 0·57N. The solution was evaporated *in vacuo* on a water bath at room temperature until 90 ml of the solvent had been removed. The concentration of the solution was re-determined and a calculated amount of methanol was added to bring it to 1-Normal ready for use.

β-[5, 6-^{3}H] *Cholestanol acetate*

Cholesterol (5 g) was refluxed for 1 hr with freshly distilled acetic anhydride (7·5 ml). On cooling, the crystals of cholesteryl acetate separated out and were washed on a Buchner funnel with ice cold methanol and dried. M.P. = 114–115°. Yield = theoretical.

Recrystallization from absolute ethanol gave crystals melting sharply at 115·5°.

Cholesteryl acetate (1·39 g) was dissolved in anhydrous redistilled ethyl acetate (17 ml), to which one drop perchloric acid (70%) and 25 mg Adams platinum black catalyst were added. Hydrogenation was carried out with tritium gas (2 mc) for 1½ hr.

The mixture was cooled to room temperature, neutralised by the addition of a predetermined quantity of 50% NaOH solution, and filtered. The filtrate was evaporated to dryness under N_2 on a water bath at 37°, and the residue recrystallised from methanol M.P. = 105–106°.

*N-Acetyl-*D*-penicillamine*

Prepared according to method given in *Biochemical Preparations*, Vol. 3, p. 112.

Cysteamine hydrochloride. L. Light & Co. Ltd.

Cystamine. Cysteamine hydrochloride (110 mg) was brought to a pH of 8·5 with a normal solution of NaOH and made up to a volume of 4·5 ml with water. Oxygen was bubbled for 3 hr through the solution heated on a water bath. At the end of this time, the product gave no reaction with N-ethylmaleimide which indicated the complete oxidation of the —SH group to the —SS group. This solution was adjusted to a pH of 7·4 with N HCl before injection.

D-*Penicillamine.* Distillers Co. Ltd.

Cysteine hydrochloride. L. Light & Co. Ltd.

Nitrogen mustard. Synthesized from formaldehyde, formic acid and diethanolamine, as detailed in the synthesis of the tritium labelled material.

METHODS

*Determination of Minimum LD*100 *dose*

The mice used were SAS original or C3H, aged 10–12 weeks. The mice were kept in groups of 10 to the cage with a plentiful supply of food and water. Solutions of HN2 in saline were injected subcutaneously, the volume of the dose not exceeding 0·2 ml per mouse. Intramuscular injection of the HN2 in the hind legs led to tissue damage and was abandoned as it prevented the mice from reaching for their food, and some of the deaths would be due to starvation and not to HN2 toxicity. Three groups of 10 mice each were used at each dose level. The dose was varied by 0·3 mg/kg of mouse between the different groups. The minimum dose level at which all animals in a group (i.e. 30) died between 4 and 10 days after the injection was the dose of HN2 used in the protection experiments.

Protection experiments

The compounds to be tested as protective agents were dissolved in water, adjusted to a pH of 7·0 $\pm$ 0·5 and injected intraperitoneally into the mice 4 hr, 1 hr, $\frac{1}{2}$ hr or $\frac{1}{4}$ hr before the injection of the HN2. The total volume of the solution injected was limited to 0·2 ml. A control group in which 0·2 ml saline was substituted for the protective compound was run with every experiment.

Protection by a compound was indicated as statistically significant when there were at least three more animals per ten animals, alive in the protected group than in the control groups at the end of 14 days.

In vitro *reactions of protective compounds with HN*2

The protective compound and HN2 were buffered with 1·5 M KH_2PO_4 adjusted to pH 7 with N NaOH. The reaction between 0·01 mmole of the two compounds was carried out in a water bath at 37°. The extent of the reaction was measured by removing samples of the reaction mixture and estimating the free —SH groups by the method of Mason (1930).

A control of the protective compound was run at the same time with buffer substituted for the HN2. This also indicated the stability of the protective compound at pH 7·0 under the conditions of the reaction.

Different molecular ratios of the HN2 to protective compound were studied, viz. 1:1; 1:2; 2:1.

Chromatography of the reaction products between protective compounds and HN2

At the end of the reaction between the protective compound and HN2, samples of the reaction products were chromatographed on Whatman No. 1 paper by descending chromatography. The solvent systems used were (a) Phenol saturated with water, and (b) Butan-1-ol–acetic acid–water (4:4:1, by volume). At the end of the run of 16 hr, the chromatograms were dried, dipped into a 0·2% solution of ninhydrin in acetone, and heated in an oven at 70° for a few minutes until the colour was developed.

Methods of radioassay

Measured samples of mouse urine were collected from the metabolic experiments, transferred to the counting vials and evaporated to dryness at 85–90°. One millilitre of hyamine was added and if the urine residue did not dissolve immediately, it was heated at 40–50° for 1 hr with the vials capped. The phosphor (10 ml) consisting of 5 g/l. of 2,5-diphenyloxazole and 0·3 g/l. of 2,2-phenylenebis (5-phenyloxazole) in toluene, was added to the sample and counted in a Tri-Carb Liquid Scintillation Spectrometer. Each sample was counted for 10,000 counts to obtain a standard error of 1 per cent. An internal standard consisting of the β-[5,6-^{3}H] cholestanol acetate (0·1 ml) dissolved in toluene was added to each vial, which was then recounted. The count was thus corrected for quenching due to hyamine and any colour in the solution.

Metabolic experiments

Four mice, constituting one group, were placed in a metabolic cage. Four different cages were used for each of the experiments. The mice in each group were injected as follows. The first group was a control and was given 0·2 ml saline 1 hr before [^{3}H] HN2 (5·5 mg/kg of mouse). The remaining three groups received 3 mg cysteamine by intraperitoneal injection, $\frac{1}{4}$ hr, 1 hr and 2 hr before the injection of [^{3}H] HN2 at the control group dosage level. The mice were allowed free access to water only, during the period of the experiment.

The urine and washings from each cage were collected at the end of 24 hr and filtered. The filtrate was concentrated to dryness under vacuum and the residue made up to 10 ml with water. After centrifugation of the solution, 1 ml samples from each group were transferred to the sample vials and counted as detailed in the method of radioassay.

A standard consisting of 0·1 ml [^{3}H] HN2 was diluted with 0·9 ml water and counted in the same manner as the urines above. The counts from the standard were used to calculate the total counts given per animal.

RESULTS

LD100 dose of HN2

The minimum LD100 figures were arrived at after a series of several experiments. It was found important to determine accurately the minimum LD100 dose within the limits of biological variation. Deviations from this dose of $\pm$0·3 mg/kg of mouse gave erratic results. This fact can be seen from the results in Table 2, where doses of HN2 less than the minimum LD100 were given.

Table 1 shows the wide difference in sensitivity to HN2 between the strains, SAS and C3H mice. The sexes also show a difference in sensitivity to HN2.

TABLE 1. MINIMUM LD100 DOSE OF HN2

Strain	Sex	Age in weeks	Dose in mg/kg of mouse
SAS	Male	8–10	4·6
SAS	Female	8–10	4·0
C3H	Male	8–12	5·5

Protection experiments

Preliminary experiments showed that if cysteamine or cystamine were given one or more hours before, or immediately after HN2, no protection was observed.

The most convenient time interval between the administration of the protective compounds and the HN2 was found to be 30 min. All protection experiments listed in Table 2 were therefore carried out by injecting the protective compound 30 min before injecting the HN2. Controls, consisting of mice injected with 0·2 ml saline

TABLE 2. PROTECTION EXPERIMENTS

Protective compound and dosage per mouse	Strain of mice	Dose of HN2 in mg/kg of mouse	No. of animals	Survival at end of 14 days
D-Penicillamine 10 mg	SAS	4·6	10	0
D-Penicillamine 5 mg	SAS	4·6	10	0
(Control)	SAS	4·6	20	0
D-Penicillamine 5 mg	SAS	4·0*	30	4
D-Penicillamine 2·5 mg	SAS	4·0*	30	3
N-Acetyl-D-Penicillamine 5 mg	SAS	4·0*	32	4
N-Acetyl-D-Penicillamine 2·5 mg	SAS	4·0*	28	6
(Control)	SAS	4·0*	30	6
Cysteine 10 mg	SAS	4·6	10	1
Cysteine 5 mg	SAS	4·6	10	0
(Control)	SAS	4·6	20	0
Cysteamine 3 mg	SAS	4·0*	20	17
Cysteamine 3 mg	SAS	4·5*	10	7
Cysteamine 3 mg	SAS	5·0*	20	7
Cysteamine 3 mg	SAS	5·5	10	4
(Control)	SAS	4·0*	20	3
(Control)	SAS	4·5*	10	1
(Control)	SAS	5·0*	20	0
(Control)	SAS	5·5	10	0
Cystamine 3 mg	SAS	4·0*	10	9
Cystamine 3 mg	SAS	5·0*	10	4
(Control)	SAS	4·0*	10	0
(Control)	SAS	5·0*	10	0
Cysteamine 3 mg	C3H	5·5	30	27
(Control)	C3H	5·5	20	0
Cystamine 3 mg	C3H	5·5	20	12
(Control)	C3H	5·5	20	0

* Doses of HN2 below the minimum LD100.

TABLE 3. CHROMATOGRAPHY OF THE REACTION PRODUCTS BETWEEN THE PROTECTIVE COMPOUNDS AND HN2

	Solvent A Rf values and colours developed with ninhydrin			Solvent B Rf values and colours developed with ninhydrin			
D-Penicillamine	0·71, violet	—	—	0·045, pink	0·08, violet	—	—
D-Penicillamine + HN2	0·71, violet	0·93, pink	—	—	—	0·113, purple	0·17, violet
Cysteamine	0·352, blue	—	0·874, purple	—	—	0·08, purple	0·27, purple
Cysteamine + HN2	0·352, blue	0·526, pink	0·874, purple	0·02, pink	0·036, purple	0·08, purple	0·27, purple
Cysteine	0·143, pink	0·31, blue	—	—	0·057, violet	—	—
Cysteine + HN2	0·143, pink	0·31, blue	0·69, pink	0·034, pink	0·057, violet	—	—

Solvent A: Phenol saturated with water.
Solvent B: Butan-1-ol–acetic acid–water (4:1:5, v/v).

instead of the protective compound, were run with each experiment. Only male mice were used in the study on protection.

The critical nature of this time interval between the administration of the protective compound and the HN2 was found to be in agreement with the results of Thirkelsen.[5]

Chromatography of the products obtained by the reaction between HN2 and cysteine, D-penicillamine, and cysteamine

The formation of at least one new product of reaction between HN2 and the protective compounds was observed by paper chromatography (Table 3).

In vitro *reactions of HN2 with three of the protective compounds*

Figures 1, 2 and 3 show results of the reactions between HN2 and D-Penicillamine, Cysteamine and Cysteine. Controls, with the HN2 omitted from the reaction mixtures are not shown, as there was no change in the optical density of each of the three compounds examined during the times of the reactions. This indicated that D-penicillamine, cysteamine and cysteine were stable over the period of the reaction. The optical density measurements were made using the reaction mixture with the protective compounds omitted, as the blank. The ordinates represent the optical density of the blue colour of the reaction mixture obtained by the ferric ferrocyanide reaction, (Mason[17]).

DISCUSSION

The metabolic fate of ^{14}C HN2 in normal and leukaemic mice has been studied by Skipper, *et al.*[18] These authors found that 15 to 20 per cent of the ^{14}C label was lost from the mice as CO_2, and only 5–10 per cent was recovered in the urine in the first 24 hr. In order to avoid such losses due to CO_2 it was thought necessary to label HN2 with tritium in the N-methyl position, which would lead to a better recovery of excreted radioactivity after administration of the radioactive HN2. Table 4 shows that a maximum of 95 per cent of the total radioactivity administered to the mice was recoverable from the urine.

TABLE 4. RECOVERY OF RADIOACTIVITY FROM THE URINES OF PROTECTED AND UNPROTECTED ANIMALS

Group	Total radioactivity injected per 4 mice in counts/min	Radioactivity recovered counts/min	% Recovery of radioactivity
1. Control. Saline before HN2	18,420	11,830	64·2
2. Cysteamine ¼ hr before HN2	18,840	15,340	81·5
3. Cysteamine ½ hr before HN2	17,740	16,880	95·0
4. Cysteamine 2 hr before HN2	19,350	10,430	54·0

An interesting point of organic chemistry arose from the specific labelling of the N-methyl group of HN2 with tritium. During the reductive methylation of the N atom containing the active hydrogen of diethanolamine with a mixture of formaldehyde and formic acid, the problem of which of these two reactants would act as the hydrogen donor was resolved. A preliminary synthesis using tritiated formaldehyde

did not result in radioactive HN2. However, when tritiated formic acid was used, the HN2 was found to be radioactive proving that in this case formic acid was the hydrogen donor for this reduction. Staples and Wagner[19] have recorded several instances where formaldehyde acts as the hydrogen donor in similar reductions utilizing a mixture of formaldehyde and formic acid.

Cysteamine has been shown to be effective in protecting an animal against the lethal action of HN2 (Thirkelsen[5, 6]). Cysteamine however, is itself toxic to the animal and a search has been made amongst a wide range of similar compounds which give the protective action without the accompanying toxicity. D-Penicillamine and its acetylated derivative were two of the compounds which were tested in this study for such a specific action. Table 2 shows that they both failed to give protection against HN2 and that cysteamine and cystamine were the only two compounds of the group which gave a significant protection. It is interesting to note that cystamine, the oxidized product of cysteamine, is the only other compound giving protection approaching that of cysteamine. Hitherto, study of a large group of compounds led Thirkelsen[5, 6] to believe that, for a compound to have protective action it should have a free SH group. This does not seem to be essential in the case of cystamine which has no free SH group though the disulphide group could easily be reduced *in vivo* to the SH group. This finding of the protective action of cystamine seems inconsistent with the theory of simple chemical inactivation as the mechanism of protective action put forward by Salerno and Friedell[11]. These authors claimed that since the animal was first flooded with a compound which reacted with HN2, the HN2 was inactivated before it had a chance of exerting its toxic action. The rapid inactivation of HN2 by the SH group of cysteamine in aqueous solution has been demonstrated by Weissberger, Heinle and Levine.[20] Since cystamine has no free SH groups, there is less likelihood of it reacting rapidly with HN2. Also, if a mixture of cystamine and HN2 was injected into mice, the reduced cystamine would inactivate at least some of the HN2 present and the toxicity of such a mixture would be expected to be less than that of HN2 alone. Preliminary experiments conducted in our laboratory showed that this was not the case, and a mixture of cystamine and HN2 was found to be as lethal as the HN2 alone.

Figures 1, 2 and 3 show that rapid disappearance of the free SH group of D-penicillamine, cysteamine and cysteine occurs, when reacted in aqueous solution with HN2. It seems therefore, that these reactions with HN2 occur at the SH groups of such compounds.

Table 3 shows the appearance of at least one new compound formed by the reaction between equimolar proportions of HN2 and the D-penicillamine, cysteamine and cysteine. The paper chromatography of the products obtained by the reaction between equimolar proportion of HN2 and the compounds was done at the end of the reactions shown in Fig. 1, 2 and 3. The formation of the complexes between the compounds and HN2 confirmed that the disappearance of the SH groups was not due to a straightforward oxidation of the SH groups, as it was attended by the appearance of these new complexes.

The slopes of the curves in Fig. 1, 2 and 3 seem to indicate that the rate of reaction between HN2 and the three compounds was highest with cysteine, lowest with cysteamine, and intermediate with D-penicillamine. This finding also argues against the inactivation theory, as the most reactive compound, cysteine, is the least protective

against HN2, and cysteamine, which is the least reactive, affords the most protection against HN2.

Goldenthal *et al.*[10] and Thirkelsen[5] have shown cysteine to protect against HN2. In our hands cysteine showed no protective action (Table 2). This may be explained by the dose used in this study (500 mg/kg of mouse) being half that used by the above authors.

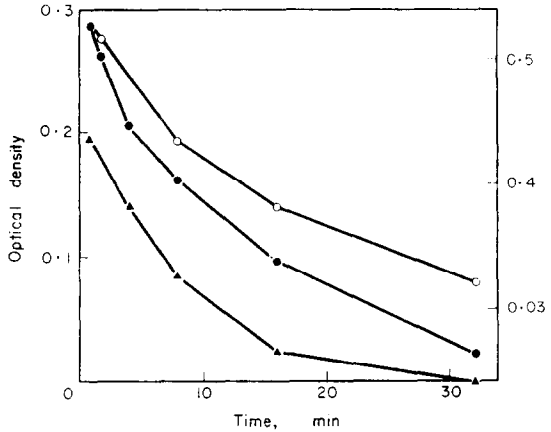

FIG. 1. Reaction between HN2 and D-penicillamine: ○, 0·01 mmole HN2 + 0·02 mmole D-penicillamine; ●, 0·01 mmole HN2 + 0·01 mmole D-penicillamine; ▲, 0·02 mmole HN2 + 0·01 mmole D-penicillamine. The optical density was obtained by the colour reaction of SH compounds (Mason[17]) The left-hand ordinates represent the optical density of ●, and ▲; and the right-hand ordinates that of ○.

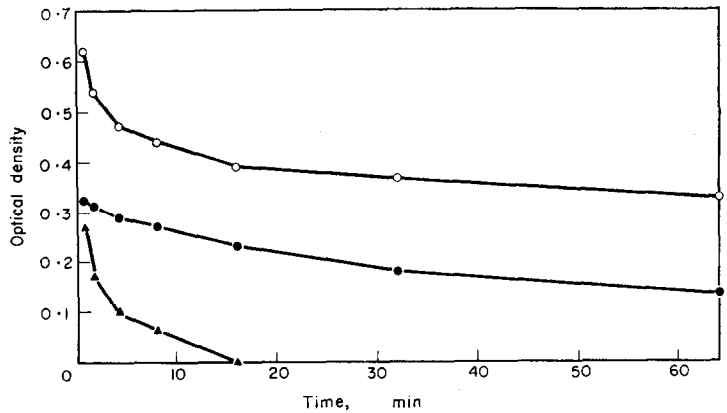

FIG. 2. Reaction between HN2 and cysteamine: ○, 0·01 mmole HN2 + 0·02 mmole cysteamine; ●, 0·01 mmole HN2 + 0·01 mmole cysteamine; ▲, 0·02 mmole HN2 + 0·01 mmole cysteamine. The optical density was obtained by the colour reaction of SH compounds (Mason[17]).

Edljarn and Pihl[21] found that if cysteamine or cystamine was injected into mice, a large proportion of the compounds was bound to the serum proteins and haemoglobin of the animals. This finding led these authors to advance the theory that cysteamine and cystamine protected the vital SH groups in the organism by the formation of

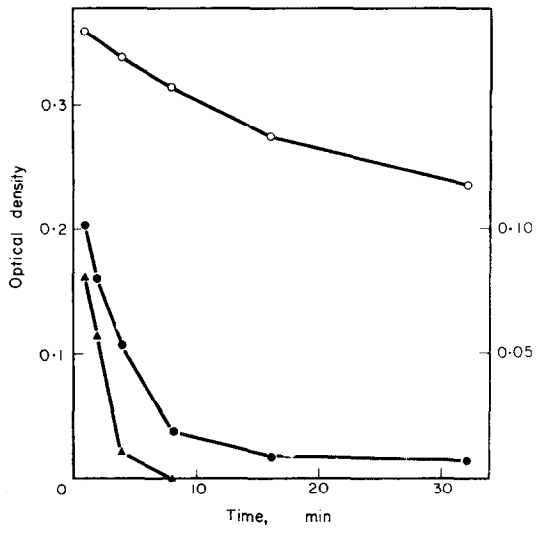

FIG. 3. Reaction between HN2 and cysteine: ○, 0·01 mmole HN2 + 0·02 mmole cysteine; ● 0·01 mmole HN2 + 0·01 mmole cysteine; ▲, 0·02 mmole HN2 + 0·01 mmole cysteine. The optical density was obtained by the colour reaction of SH compounds (Mason[17]). The left-hand ordinates represent the optical density of ○, and ●; and the right-hand ordinates that of ▲.

mixed disulphides with the SH group of the proteins and thus blocking the point of attack by the HN2. Burnop *et al.*[22] have shown that the reaction between HN2 and proteins is very rapid, and does not take place at the free NH_2 groups. The protein complex with cysteamine or cystamine seems to dissociate by the end of 1 hr. This probably explains the reason for protection by cysteamine or cystamine only lasting for 1 hr before injection of the HN2. Thirkelsen[5] has shown the metabolism of cysteamine to be nearly complete at the end of 1 hr.

The results obtained in our study seem to be consistent with what one would expect on the basis of the observations and theory of Eldjarn and Pihl.[21] Table 4 shows that the maximum recovery of injected radioactivity occurs when cysteamine is injected $\frac{1}{2}$ hr before the HN2. This is probably the time when the protection given by the cysteamine has reached its peak, as the recovery figure at the $\frac{1}{4}$ hr period is less. This seems to indicate that since the HN2 is prevented from attaching itself to its normal sites, which are blocked by the disulphide bridges between cysteamine and the proteins, more of it gets excreted than if it were allowed to take its normal course. When the interval between the injections is 2 hr, the recovery of radioactivity is actually less than in the control, this suggests that the cysteamine–protein complex no longer exists, the HN2 is now free to attach itself to the protein and therefore less radioactivity is excreted.

The observations recorded in this study argue against the theory of simple chemical inactivation being the mechanism of the protective action of cysteamine against HN2. They favour the theory of Eldjarn and Pihl[21] as being the more likely explanation of this protective action. It is important that studies of this nature be extended as the key to the protective mechanisms may provide the solution to the problem of the mechanism of the action of HN2 itself.

SUMMARY

1. [^{3}H] Methyl-di-(2-chloroethyl) amine has been synthesized.
2. The protection of mice by previous administration of chemical compounds related to cysteine against the lethal action of HN2 has been studied.
3. Of the compounds studied (cysteine, cysteamine, cystamine, D-penicillamine, N-acetyl-D-penicillamine) only cysteamine and cystamine were found to give significant protection.
4. The rate of reaction between HN2 and cysteine, D-penicillamine and cysteamine has been studied.
5. Chromatographic analysis has shown the formation of complexes between HN2 and these three compounds.
6. The excretion of radioactivity was found to be higher in the cysteamine protected group of mice than in the unprotected group after administration of tritiated HN2.
7. The mechanism of this protective action of cysteamine is discussed.

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