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REDUCTION OF THE TOXICITY OF 'RADIOMIMETIC' ALKYLATING AGENTS IN RATS BY THIOL PRETREATMENT—III.

THE MECHANISM OF THE PROTECTIVE ACTION OF THIOSULPHATE

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Abstract—Thiosulphate affords good protection against the toxicity of HN2 but none against the toxicity of an aromatic nitrogen mustard Merophan. Cysteine, however, gave a good protection against the toxicity of both these mustards. These results can be explained on the basis of the different mechanisms by which the aliphatic and aromatic nitrogens mustard alkylate. Mixtures of thiosulphate and cysteine gave good protection against HN2 toxicity but little against Merophan, which suggests that thiosulphate may prevent cysteine from entering cells.

CYSTEINE is very effective, in rats, in reducing the toxicity of some nitrogen mustards. The extent of this protection has been shown to depend on, (a) the time interval between the administration of the thiol and the mustard, (b) the amount by which cysteine increases the protein free thiol content of tissues, and (c) the chemical reactivity of the mustard.[1,2] Although cysteine may be expected to have many pharmacological effects *in vivo*, all the known facts concerning the mechanism of this protection can be explained by assuming that protection occurs as a result of an intracellular reaction between the added thiol and the nitrogen mustard. As a result of this reaction, less mustard reacts with normal cell constituents with a corresponding reduction in toxicity. Thiosulphate is more reactive to mustards *in vitro* than cysteine; and although it can be given to rats at very high dose levels, it gave no protection against the toxicity of Merophan (*o*-di-2-chloroethylamino-DL-phenylalanine).[1]-This finding can be explained if we assume that mustards must enter cells and alkylate intracellular constituents in order to be toxic. Thiosulphate does not penetrate cells and remains in the extracellular fluid.[3] Competitive removal of aromatic nitrogen mustards in the extracellular fluid cannot occur since they react by an SN_1 mechanism.[4] Oh the other hand, certain aliphatic nitrogen mustards such as HN2 (methyl di-2-chloroethylamine) react by an SN_2 mechanism,[4] and may be expected to react extensively with thiosulphate present in the extracellular spaces. Consequently less HN2 will enter the cells, and a reduction in toxicity might be expected in rats exposed to HN2. The protection given by thiosulphate and cysteine against Merophan (an SN_1 reactor) has been compared with the protection these two compounds give against the toxicity of HN2 (an SN_2 reactor).

MATERIALS AND METHODS

Eight-week old female rats, 200–250 gm in weight, from an inbred colony, were used. They were maintained on rat cake and water *ad lib.* $LD_{50,8}$ were calculated, using the method of Weil,[5] and each determination was the mean of at least two experiments using ten animals at each dose level. The compounds were prepared for injection by dissolving in water (or water containing an equivalent of sodium hydroxide for L-cysteine hydrochloride) and injected immediately. Merophan was dissolved in methanol and injected as an aqueous solution containing 5% methanol. For the combination experiments, using cysteine and thiosulphate, the cysteine hydrochloride was prepared for injection in the usual manner and solid sodium thiosulphate added. Five minutes or less elapsed between the addition of the thiosulphate to the cysteine solution and the completion of injection of the animals. The thiols were used at the maximum tolerated dose estimated previously,[1] 1 g/kg for L-cysteine hydrochloride and 2 g/kg for sodium thiosulphate pentahydrate. All injections were intraperitoneal, except where otherwise stated.

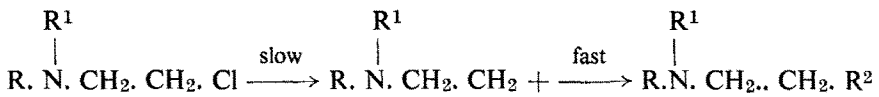
RESULTS

The mustards were given at least thirty minutes after the thiol to avoid any local reaction in the peritoneum. The results listed in Tables 1 and 2 show that, for HN2, protection is best if cysteine or thiosulphate is given 30 min before the mustard. There is a fall-off in the extent of protection, as the time-interval between injection of cysteine or thiosulphate and mustard increases. After the intraperitoneal injection of 1 g/kg of cysteine, many tissues show a rise in the level of intracellular protein free SH[2]. In all cases this rise in SH reaches its peak in about 30–45 minutes (i.e. when protection against HN2 or Merophan is best) and returns to normal in about $2\frac{1}{2}$ hr If either HN2 or Merophan is given later than $2\frac{1}{2}$ hr after the cysteine (i.e. when the SH level of the tissue is back to normal), there is no protection. For both HN2 and Merophan the degree of protection given by cysteine would therefore seem to be related to the level of free SH in tissues (it has yet to be established whether this rise in SH is due to cysteine accumulation, or, whether the cysteine induces increased level of some other thiol.) Similarly, the protection obtained against HN2 with thiosulphate appears to be related to its blood concentration. After the intraperitoneal injection of thiosulphate the blood concentration is high at 30 min and falls to zero in $2\frac{1}{2}$ hr. Protection against HN2 is good when the thiosulphate is given 30 min before the HN2, while little protection is obtained if more than 2 hr is allowed to elapse between injection of the thiosulphate and HN2. Table 3 gives the dose reduction factors obtained for Merophan and HN2 (i.p. or subcutaneous) in animals pretreated with cysteine, thiosulphate or a combination of these two agents 30 min beforehand. The dose reduction factor of 5·2 obtained for animals pretreated with cysteine and given HN2 subcutaneously, is in good agreement with the dose reduction factor of 6·0 obtained by Brandt and Griffin[6] under similar conditions. Hatiboglu[7] has previously demonstrated that sodium thiosulphate can reduce the lethality of HN2 in mice but no dose reduction factor was obtained. Thiosulphate gave no protection against Merophan but a good protection against HN2 (especially when the HN2 was given subcutaneously). Against HN2 the combination of thiosulphate and cysteine gave a better protection than that observed with either thiol alone. Whether this is an additive effect requires further investigation, A surprising finding was that, against

Merophan, the combination of thiosulphate and cysteine did not give the protection which would have been expected from its cysteine content alone (i.e. a dose reduction factor of 4·2), but a greatly decreased protection. In fact, the dose reduction factor of 1·5 obtained with the mixture is only the protective effect which would be expected from 225 mg/kg of cysteine alone.

DISCUSSION

The aromatic nitrogen mustards react by an SN_1 mechanism; that is, their *rate* of alkylation depends entirely on the rate at which they form the carbonium ion (I). This rate is not influenced by the presence of the molecules capable of alkylation, since the rate determining step in the reaction is ionization to form the carbonium ion.



(I)

Certain aliphatic mustards such as HN2 alkylate by an SN_2 mechanism. In this case the *rate* of reaction depends on the concentration of both the reacting substances (i.e. the mustard and the centre which it alkylates). The higher the concentration of centres which react with HN2, the greater will be the rate of reaction of HN2. The fact that the rate of reaction of HN2 can be influenced by the concentration of reacting centres while the rate of reaction of Merophan is unaffected can explain why thiosulphate protects against HN2 toxicity but not against Merophan.

TABLE 1. THE PROTECTIVE ACTION OF THIOSULPHATE AGAINST THE LETHALITY OF HN_2 HYDROCHLORIDE (I.P.) IN RATS

Time of Thiosulphate Pretreatment (2g/kg; i.p.)	HN_2 LD_{50} mg/kg	D.R.F.*
None	1·28	1·0
30 min before	4·06	3·2
45 min before	3·53	2·8
1 hr before	2·83	2·2
2 hr before	1·87	1·5
5 Hr before	1·30	1·0

* Dose reduction factor. The ratio (LD_{50} HN_2 (or Merophan) after thiol pretreatment)/(LD_{50} HN_2 (or Merophan) alone).

If one considers HN2 after intraperitoneal injection passing from the peritoneum into the blood stream and thence via the interstitial fluid into cells, then a high concentration of thiosulphate in these extracellular spaces will increase the rate of reaction of HN2 (and therefore its deactivation) before it reaches the cell. Therefore, when thiosulphate is present in the extracellular fluid HN2 will react to a greater extent extracellularly than it would normally. If one assumes that it is intracellular alkylation that is responsible for toxicity, then, when thiosulphate is present in the extracellular fluid the greater amount of reaction of HN2 that takes place extracellularly will

mean that less HN2 will enter cells with a corresponding reduction in toxicity. With Merophan, however, this increased rate of extracellular reaction in the presence of thiosulphate will not occur (since Merophan alkylates by an SN_1 mechanism). Although in the extracellular fluid Merophan may react with thiosulphate rather than with, say, protein, which might be the normal site of extracellular reaction, the *rate* of reaction of Merophan will be the same as if the thiosulphate were absent. As a consequence the same amount of Merophan will enter cells whether or not thiosulphate is present extracellularly. If it is inside the cell that the toxic alkylations takes place, then, as the same amount of Merophan enters in both cases the toxicity will remain unaltered.

Cysteine, which penetrates cells, will protect against the toxicity of Merophan, not by altering the rate of reaction of Merophan but by altering the sites in the cell alkylated by this mustard. When a mustard enters a cell there are many reactive sites which it can alkylate. Since these sites are in a large excess compared with the amount of mustard which enters at physiological dose levels, then only a small proportion of these sites will be alkylated. The sites alkylated will depend on (a) the competition factors of the various sites, and (b) their relative concentration. Some of these sites will not be necessary for the survival of the cells (non-essential sites), while alkylation of other sites may lead to cell death. The presence of exogenous cysteine in the cell will, in effect, increase the concentration of non-essential sites which are competing with essential sites for alkylation by the mustard. As a result, some of the mustard which normally would have alkylated some essential site (perhaps DNA) is now deactivated by reaction with cysteine. Thiosulphate is therefore protecting by increasing the rate of reaction of HN2 before it enters cells, while cysteine is protecting against Merophan by altering the intracellular sites which it alkylates.

TABLE 2. THE PROTECTIVE ACTION OF L-CYSTEINE HYDROCHLORIDE AGAINST THE LETHALITY OF HN_2 HYDROCHLORIDE (I.P.) IN RATS

Time of Thiol Pretreatment (lg/kg; i.p.)	HN_2 LD_{50} mg/kg.	D.R.F.
None	1·28	1·0
30 min before	6·33	5·0
1 hr before	5·65	4·4
2 hr before	<4·0	<3·1

Cysteine may protect against the toxicity of HN2 in two ways. Firstly, if present in the extracellular fluid it would increase the rate of reaction of HN2 and so decrease the amount entering cells. Secondly, inside the cell it would restrict the alkylation of essential sites. The poor protection observed with the mixture of thiosulphate and cysteine against Merophan toxicity, in contrast to the excellent protection this mixture affords against HN2 toxicity (the dose reduction factor of about 9·0 is the highest factor so far obtained in animal protection experiments) was an unexpected finding. Certainly it is known that thiosulphate reacts with cysteine to form H_2S, cystine and sulphate as major products,[8] but in our experiments, this reaction, judged by formation

of H_2S and cystine, was not complete until at least 24 hr after mixing. In the protection experiment the injection of the cysteine–thiosulphate combination was completed within 5 min after mixing, when no cystine had been precipitated and very little H_2S evolved. In any case, since the mixture is still protective to HN2 it is unlikely that there has been any destruction of the cysteine or thiosulphate. A possible explanation is that the thiosulphate in some way prevents cysteine from entering cells. Since no rise in intracellular SH would then be expected, no protection against Merophan would occur, but there would still be good protection against HN2 because the thiol and thiosulphate concentration of the extracellular fluid would remain high. This matter is now under further investigation.

TABLE 3. THE PROTECTIVE EFFECT OF THIOSULPHATE AND CYSTEINE ON THE LETHALITY OF HN_2 HYDROCHLORIDE OR MEROPHAN

	Merophan (i.p.)			HN_2 (i.p.)			HN_2 (Subcut.)		
Pretreatment	LD_{50} mg/kg	Fiducial Limits (95 per cent)	D.R.F.	LD_{50} mg/kg	Fiducial Limits (95 per cent)	D.R.F.	LD_{50} mg/kg	Fiducial Limits (95 per cent)	D.R.F.
None	3·67	3·24 to 4·16	1	1·28	1·08 to 1·51	1	2·06	1·74 to 2·43	1
Thiosulphate 2 g/kg i.p., 30 min before	3.67	3·24 to 4·16	1	4·06	3·44 to 4·79	3·2	10·68	9·05 to 12·60	5·2
Cysteine 1 g/kg i.p., 30 min before	15·24	13·65 to 16·71	4·2	6·33	5·37 to 7·46	4·9	10·68	9·39 to 12·14	5·2
Cysteine 1g/kg + Thiosulphate 2 g/kg	5·30	4·50 to 6·23	1·4	12·27	11·31 to 13·31	9·6	17·34	15·16 to 19·84	8·4

Where attempts have been made to improve the selectivity of action of the nitrogen mustards as anti-tumour agents by thiol pretreatment, it would seem from our results that thiosulphate, since it protects by reacting with the mustard extracellularly, would offer no advantage, as where reduction in lethality took place there would be a corresponding reduction in anti-tumour action. Also, if thiosulphate is used for instance in regional perfusion experiments, as a protector of the systemic circulation it would appear only to be applicable where the mustard used reacted by an SN_2 mechanism. For cysteine, an improved therapeutic index could only be expected if the tumour took up less cysteine (or showed a smaller rise in SH content after cysteine administration) than other tissues sensitive to the mustard.

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