Biochemical Pharmacology, 1965, Vol. 14, pp. 569–577. Pergamon Press Ltd., Printed in Great Britain.

REDUCTION OF THE TOXICITY OF "RADIOMIMETIC" ALKYLATING AGENTS IN RATS BY THIOL PRE-TREATMENT—IV.

PROTECTION AGAINST BONE MARROW DAMAGE

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(*Received* 26 *October* 1964; *accepted* 26 *November* 1964)

Abstract—The nucleic acids, nucleotide and protein levels in rat bone marrow were followed after the administration of the aromatic nitrogen mustard Merophan alone and with cysteine pre-treatment. The DNA dose response curve was similar to the mortality curve, which suggests that the DNA concentration of the bone marrow can be used as a sensitive index of toxicity. Based on this index the dose reduction factor for bone marrow toxicity given by cysteine pre-treatment agreed with that for whole body protection. A direct relationship was found between the rise in free SH in the bone marrow after administration of various doses of cysteine and the protection afforded to this tissue. The mechanism of the protective action thus appears to be that of an intracellular reaction between a thiol and the nitrogen mustard.

RATS can be protected against the toxicity of certain nitrogen mustards if the animals are pre-treated with cysteine.[1–3] This whole body protection is related to the intracellular rise in free SH which occurs in organs (spleen, liver, thymus) after administration of cysteine,[4] and it has been suggested that protection is a result of an interaction between the free SH in cells and the mustard. In order to establish more precisely the relationship between cysteine protection and the free SH level of tissues, we have attempted to correlate the rise in free SH which occurs in one particular tissue (bone marrow), after administration of various doses of cysteine, with the extent to which this tissue is protected against the toxicity of an aromatic nitrogen mustard, Merophan (*o*-di-2-chloroethylamino-DL-phenylalanine). A quantitative measure of bone marrow toxicity was required, and since alkylating agents have been shown to have an effect on the DNA concentration of tissues,[5, 6] the effect of Merophan on the DNA, RNA, nucleotide and protein concentration of bone marrow has been examined to determine whether effects on any of these fractions can be used quantitatively as a measure of bone marrow toxicity.

MATERIALS AND METHODS

Female Wistar rats from an inbred colony, weighing 200–250 g were used. They were allowed water and rat cake ad libitum. Merophan was prepared for injection by dissolving in methanol and then adding water to make a 5% solution of methanol in water. Cysteine hydrochloride was dissolved in water and neutralised with one equivalent of sodium hydroxide. All injections were intraperitoneal. Solvent controls

for Merophan injections were 5% aqueous methanol solutions and for cysteine injections, saline equivalent to that produced on neutralization of cysteine hydrochloride (e.g. 4% saline for cysteine hydrochloride 1 g/Kg, 2% saline for 500 mg/Kg). In animals treated with both cysteine and Merophan the cysteine injection was given 30 min before the Merophan.

Bone marrow from two femurs was forced with water into a small tube, made up to 5 mls and the suspension homogenised at 0° using an ultrasonic disintegrator. A two ml aliquot of the homogenate was carefully evaporated to constant weight in a weighed dish at 100° for a measure of dry weight. A further 2·5 ml aliquot of the homogenate was used for the estimation of nucleotide, DNA, RNA and protein concentration by the method of Ogur and Rosen.[7] The bone marrow homogenate was first extracted twice with perchloric acid (0·2 N) at 0°, and after centrifugation the combined supernatants were estimated for nucleotide content spectrophotometrically at 260 mμ. The solid residue was extracted with an ethanol-ether mixture (3 : 1) to remove lipids and the nucleic acid obtained from the residue by incubation for 20 min at 70° with perchloric acid (0·5 N). The perchloric acid supernatant was analysed for total nucleic acid content by reading at 260 mμ. Further aliquots were used to determine the DNA content by the Burton technique[8] and RNA by the Orcinol reaction.[9] After the hot perchloric acid extraction the residue was dissolved in NaOH (0·1 N) and the protein content measured by the method of Lowry.[10]

The free SH concentration of the bone marrow was determined by the method of Ellman.[11] Bone marrow from two femurs was blown into a weighed vessel and after weighing, 2 mls of 5% sulphosalicylic acid was quickly added. The sample was homogenised at 0° by ultrasonic disintegration and the protein precipitate removed by centrifugation. One ml of the supernatant was mixed with a freshly prepared solution consisting of 3·8 ml of phosphate buffer (0·5 M, pH 6·8 with E.D.T.A. added to make a final E.D.T.A. concentration of 10^{-3} M) and 0·2 ml of 5,5′-dithio(2-nitrobenzoic acid) (10^{-2} M in phosphate buffer). After standing for 10 min at room temperature the solution was read at 412 mμ against a reagent blank (reagent solution 4 ml and 1 ml of 5% sulphosalicylic acid). Cysteine hydrochloride in 5% sulphosalicylic acid (1 ml) and reagent solution (4 ml) was used for the SH calibration. The SH values were expressed as a concentration of the bone marrow wet weight, since all the material obtained from the two femurs was used for the estimation.

RESULTS

Based on estimation of lethality (LD_{50}), cysteine (1 g/Kg) given 30 min before Merophan gives a whole body dose reduction factor (DRF) of about four.[3] In other words, in rats pre-treated with this dose of cysteine, four times the dose of Merophan must be given to produce the same degree of toxicity as seen in rats receiving Merophan alone. 2 mg/Kg of Merophan causes a weight loss in rats and some toxic symptoms (anorexia, diarrhoea and 'harsh' fur) from which they recover within 4–6 days after treatment. No deaths have resulted from this dose but it can be considered to be a maximum tolerated dose. Since the whole body dose reduction factor is about 4, then after cysteine pre-treatment the maximum tolerated dose of Merophan should be about 8 mg/Kg and, in fact the toxicity to rats at this dose level is very similar to that seen in rats receiving 2 mg/Kg Merophan with no pre-treatment. The effect (over 12 days) of these two maximum tolerated doses on the bone marrow

concentration of DNA, RNA, nucleotides and protein is shown in Figs. 1 and 2. Also shown is the effect of 2 mg/Kg of Merophan in rats pre-treated with cysteine. This protected dose is well tolerated, giving none of the gross toxic symptoms described above. Each point in Figs. 1 and 2 is the mean of three independent determinations. Each day that determinations were made, three control animals were included and the values obtained for the treated animals expressed as a percentage of these control values. Little effect on protein concentration could be demonstrated and only a small effect on the nucleotide level (Fig. 1). However, the bone marrow

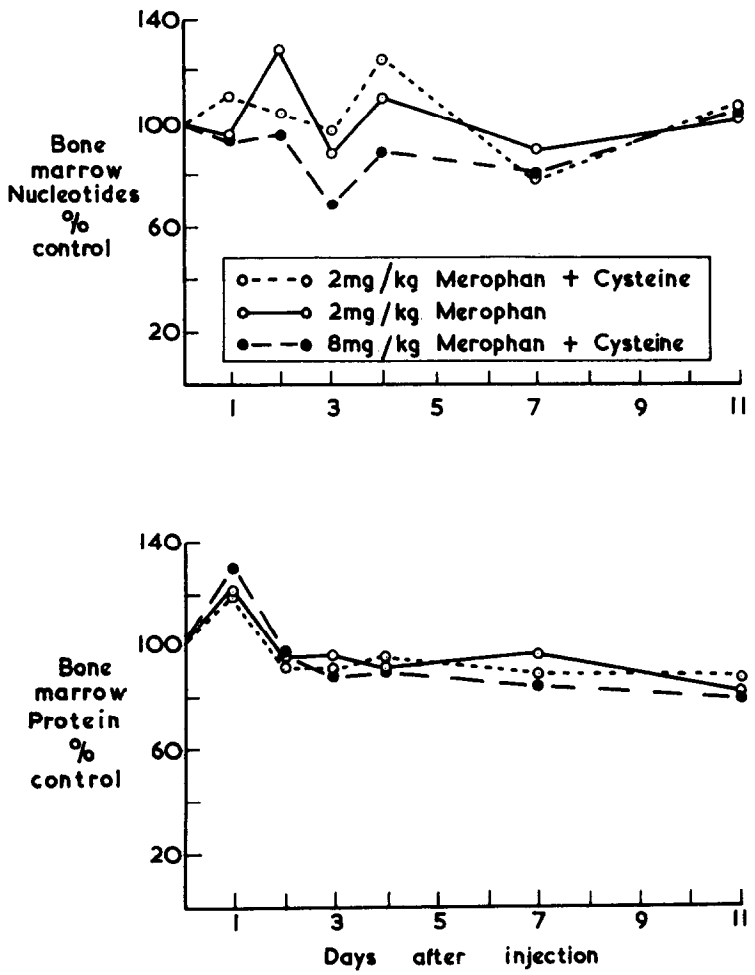

FIG. 1. The effect of Merophan on the bone marrow nucleotide and protein concentration. Control values: nucleotides 13·6 (±2·0) mg/1g dry wt. bone marrow; protein 497 (±122) mg/1g dry wt. bone marrow.

concentration of both DNA and RNA fell, reaching a minimum the second day after injection (Fig. 2). The similarity between the effects of the two maximum tolerated doses (2 mg Merophan alone and 8 mg with Merophan and Cysteine) and the lesser fall caused by the well tolerated dose of 2 mg/Kg of Merophan in cysteine

pre-treated animals, suggested that this fall was related to the Merophan dose. The effect of cysteine pre-treatment in protecting against the fall in circulating leucocytes in the blood has been described.[3] Table 1 shows the protection afforded by cysteine against the fall in blood neutrophils and the dose response in cysteine pre-treated rats.

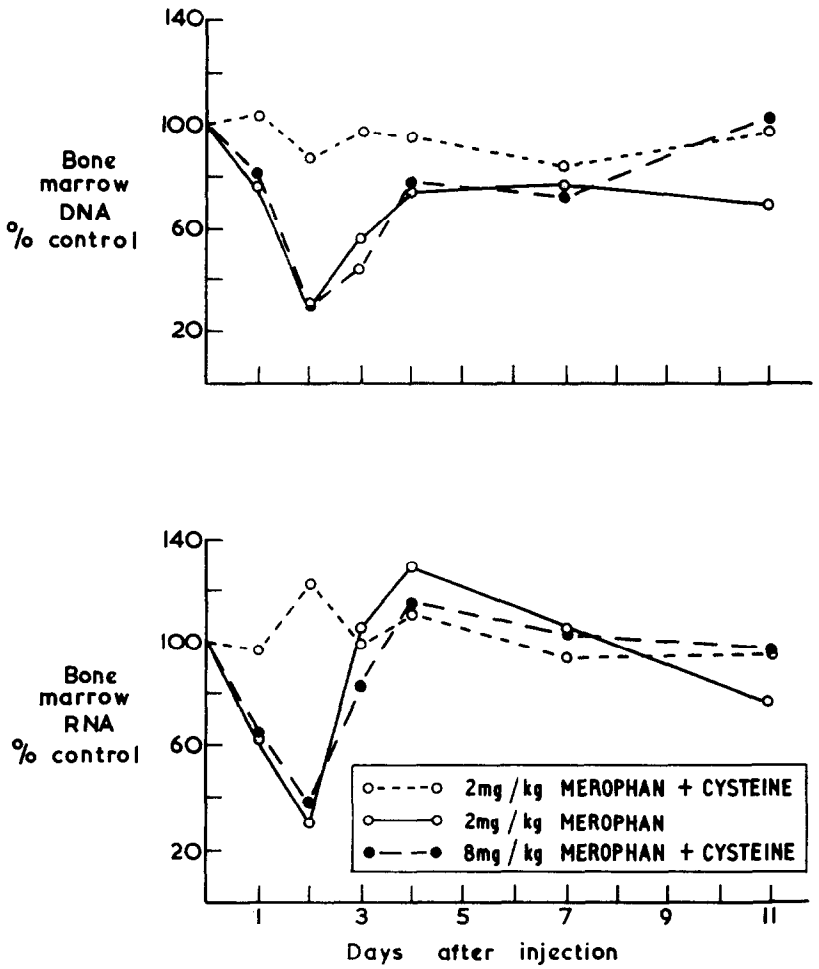

FIG. 2. The effect of Merophan on the bone marrow DNA and RNA concentration. Control values: DNA 114 (±19·8) mg/1g dry wt. bone marrow; RNA 59·7 (±13·7) mg/1g dry wt. bone marrow.

Although the time at which the minimum nucleic acid level occurs is probably dependent on the dose of Merophan used, three days after injection was considered a suitable time to measure the RNA and DNA levels of bone marrow after various doses of Merophan. At this time we have compared both the RNA and DNA levels after several doses of Merophan to determine whether a relationship exists between the fall in nucleic acid concentration and whole body toxicity. The results are shown in Fig. 3 and for comparison the dosage mortality curve for Merophan obtained in these rats in earlier experiments is also shown. The DNA dose response curve shows

TABLE 1. EFFECT OF CYSTEINE PRE-TREATMENT ON THE FALL OF BLOOD NEUTROPHILS INDUCED BY MEROPHAN

Dose of Merophan (mg/kg)	% Fall of Neutrophils*
2	85
2 + cysteine	15
4 ,,	50
8 ,,	80

* % fall from normal values at time of maximum depression (4 days after administration of Merophan).

a steep decline, which parallels closely that of the dosage mortality curve. The effect on bone marrow DNA would clearly seem to be a sensitive measure of toxicity (up to 80% fall in DNA occurs before any deaths are registered on the dosage mortality curve). Using this bone marrow DNA response curve, the effect of cysteine in reducing the action of Merophan has been studied. In Fig. 4 the effect of cysteine (1,000 and 500 mg/Kg) in reducing the fall in bone marrow DNA concentration 3 days after

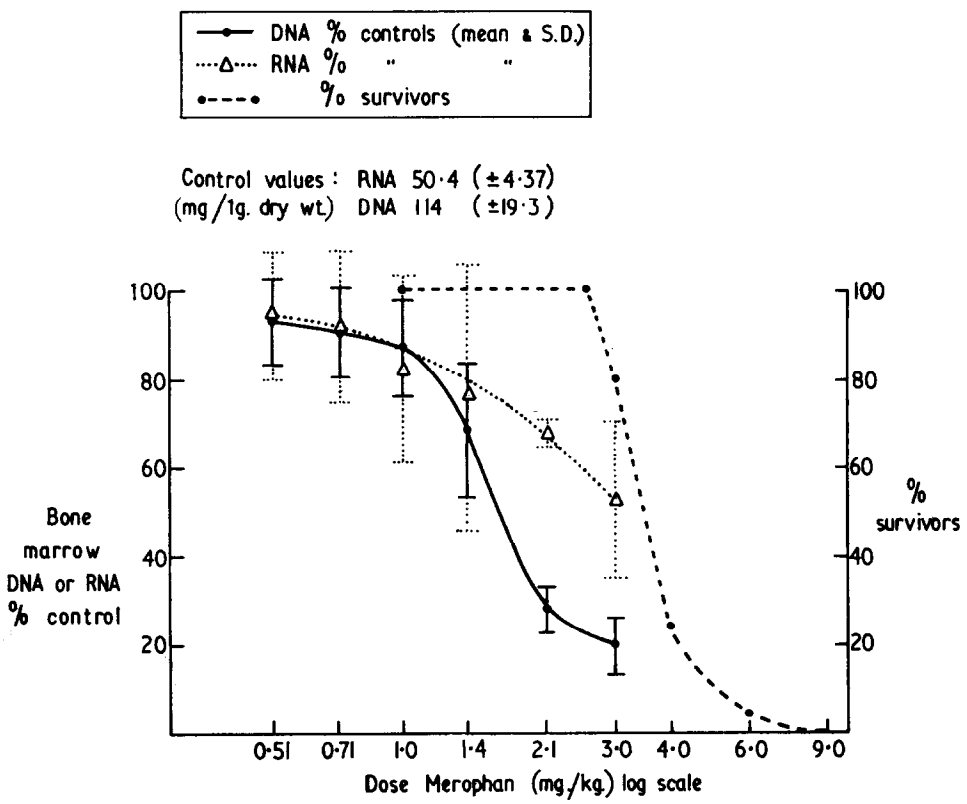

FIG. 3. Dosage mortality curve and DNA and RNA dose response curves. The DNA and RNA values were obtained three days after the administration of Merophan.

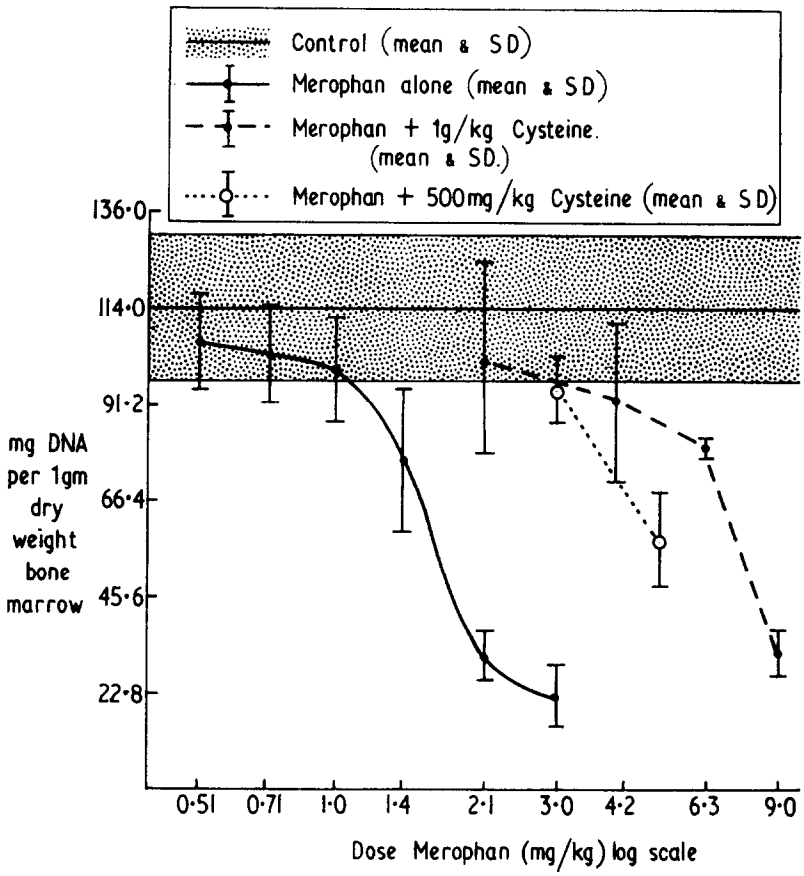

FIG. 4. The effect of Merophan on the bone marrow DNA concentration in rats receiving Merophan alone and Merophan and Cysteine (1000 and 500 mg/Kg).

injection of Merophan, is shown. The bone marrow dose reduction factor has been calculated from the ratio

$$\frac{\text{Dose of Merophan to cause 50\% DNA fall in cysteine pre-treated rats}}{\text{Dose of Merophan to cause 50\% DNA fall in rats with no pre-treatment.}}$$

Dose reduction factors for Merophan in rats pre-treated with 1000 and 500 mg/Kg of cysteine are given in Table 2. For comparison the LD_{50}'s and the corresponding whole body dose reduction factors are also recorded. Also in this table are the bone marrow free SH figures of solvent-treated control rats and rats killed 30 min after injecting different doses of cysteine. After administration of cysteine the free SH of bone marrow rises. The maximum SH value occurs 30–45 min after injection and the SH returns to normal in about 3 hr (Fig. 5). The pattern is similar to that previously reported for rat spleen.[4] Also shown in Fig. 5 are the bone marrow SH levels of rats, 30 min after receiving 500 and 250 mg/Kg of cysteine.

TABLE 2

Pretreatment	LD_{50} (mg/Kg)	D.R.F.	*50% fall in bone marrow DNA (mg/Kg)	Bone marrow D.R.F.	Bone marrow SH 30 min after cysteine (μg/g wet wt.)
None	3·67		1·7		121 (±34·5)
Cysteine 250 mg/Kg i.p.	6·50	1·8			142 (±21·5)
Cysteine 500 mg/Kg i.p.	11·02	3·0	5·0	2·9	178 (±27·0)
Cysteine 1000 mg/kg i.p.	15·24	4·2	7·4	4·4	261 (±38·5)

* The dose of Merophan to give 50% fall in the bone marrow DNA concentration.

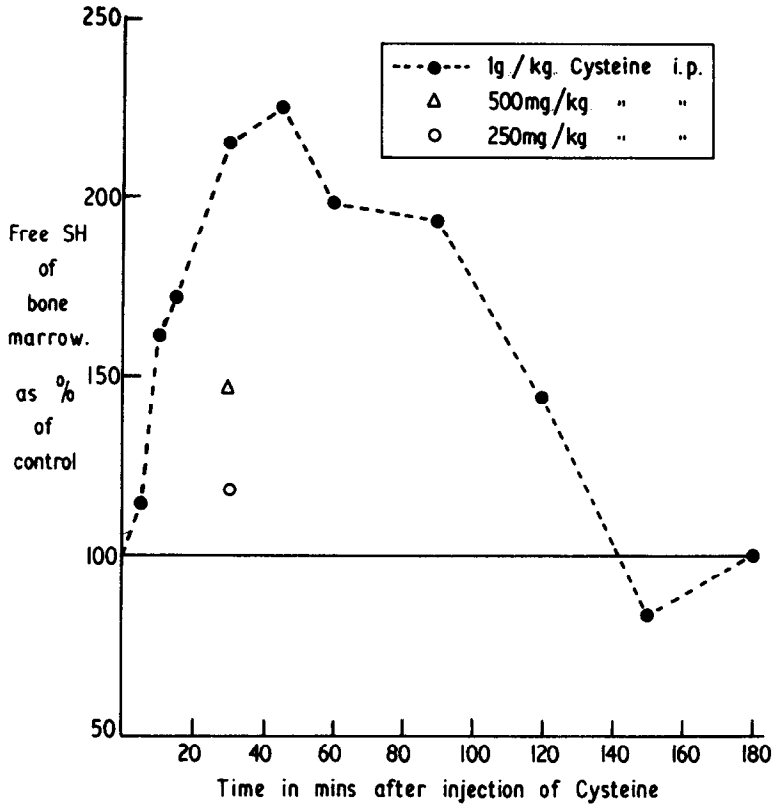

FIG. 5. The rise in bone marrow protein free SH level after administration of Cysteine.

DISCUSSION

Using the DNA concentration of bone marrow as a measure of Merophan toxicity, the protective effect of various doses of cysteine has been estimated. Expressed as a dose reduction factor, the degree of bone marrow protection given by 100 and 500 mg/Kg of cysteine is very similar to the values obtained for whole body protection, and indicates the usefulness of bone marrow DNA concentration as a measure of toxicity. It is of interest that Merophan begins to be lethal only when the bone marrow DNA

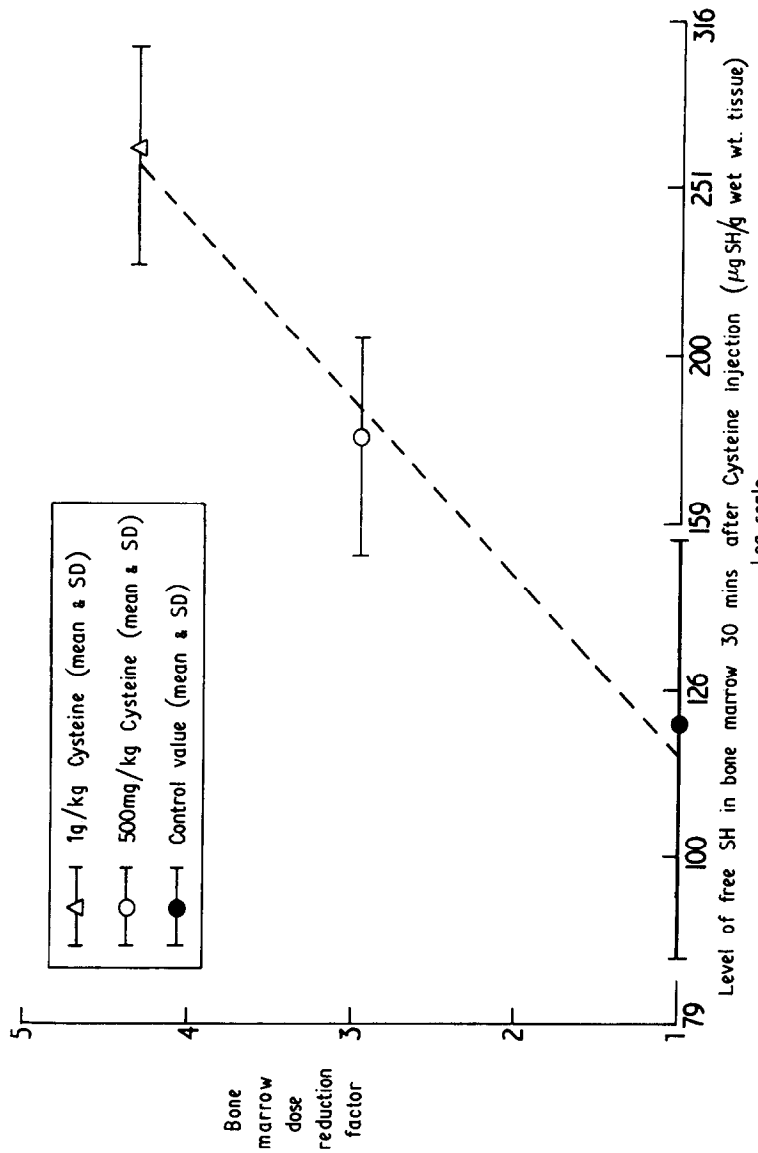

FIG. 6. The relationship between protein free SH level of bone marrow and the extent of protection given by various doses of Cysteine against Merophan toxicity.

concentration has fallen to about 20 per cent of its normal value. At this point small increases in the dose of Merophan lead to a greatly increased lethality giving the steep mortality dose response curve seen in Fig. 2. Since effects on bone marrow DNA are apparent at Merophan doses which give no lethality and no gross symptoms of toxicity, this parameter can be used as a more sensitive measure of toxicity and could presumably be used to show protection by compounds which have no effect on survival time. The fact that the DNA (but not the RNA, nucleotide or protein) dose response curve had a steep slope corresponding closely with the dosage mortality curve could imply that the toxicity of the alkylating agent is related to an effect on DNA. If the bone marrow dose reduction factors from Table 1 are plotted against the percentage rise in bone marrow SH level, thirty minutes after cysteine injection (Fig. 6), a correlation can be seen between the extent of protection and the level of free SH in the bone marrow.

This would support the hypothesis that cysteine protects tissues by increasing the amount of free SH substances in cells (cysteine itself?). These SH substances react with the alkylating agent, so preventing the alkylation of cellular sites. Cysteine itself has been shown to have pharmacological effects on tissues,[12] and it might be suggested that a specific effect of cysteine on cells is responsible for the protection, and that the rise in tissue SH after cysteine parallels this pharmacological effect, so giving the correlation shown in Fig. 5. However, from Figs. 1 and 2 it can be seen that cysteine is reducing to a similar extent the effect of Merophan on all the cellular fractions measured which are normally affected by this agent. Cysteine would appear merely to be reducing the overall potency of the alkylating agent so that in the presence of cysteine more drug is required to give the same effect. The reduced amount of drug available in cysteine pretreated animals will still react with cell constituents in the same proportion as before, giving a net effect of reduced potency of the drug. If one particular site only were protected, or if for example cysteine was stimulating regeneration rather than preventing alkylation, then a different pattern to that shown in Figs. 1 and 2 would be expected.

Acknowledgements—This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, the Public Health Service Research Grant No. CA-03188-08 from the National Cancer Institute, U.S. Public Health Service, and the award of a Wellcome Trust Fellowship to one of us (A.J.).

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