TABLE II. Protoplast Formation.

| Penicillin | | | Bacitracin | | | Novobiocin | | | Vancomycin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | NaCl | | | NaCl | | | NaCl | | | NaCl | |
| μg/ml | 1.5% | 3.0% | μg/ml | 1.5% | 3.0% | μg/ml | 1.5% | 3.0% | μg/ml | 1.5% | 3.0% |
| 620 | + | + | 20,000 | 0 | 0 | 1,000 | 0 | 0 | 1,000 | ± | + |
| 310 | + | + | 10,000 | 0 | 0 | 500 | 0 | 0 | 500 | + | + |
| 155 | + | ± | 5,000 | 0 | 0 | 250 | 0 | 0 | 250 | + | 0 |
| 78 | + | 0 | 2,500 | 0 | 0 | 125 | 0 | 0 | 125 | ± | 0 |
| 39 | + | 0 | 1,250 | + | 0 | 62 | + | + | 62 | 0 | 0 |
| 20 | ± | ± | 625 | 0 | 0 | 31 | 0 | ± | 31 | 0 | 0 |
| 10 | ± | 0 | 312 | + | + | 16 | 0 | 0 | 16 | 0 | 0 |
| 5 | ± | 0 | 156 | 0 | 0 | 8 | + | 0 | 8 | 0 | 0 |
| 2 | ± | 0 | 78 | 0 | + | 4 | 0 | 0 | 4 | 0 | 0 |
| 1 | 0 | 0 | 39 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 |

+ many protoplasts, ± few protoplasts, 0 no protoplasts

known to be L forming bacteria, casts doubt upon the hypothesis that these agents act only or primarily by interference with cell wall synthesis. The production of large spherical organisms, *i.e.*, protoplasts, does give evidence that the cell wall is attacked. The appearance of these forms is presumably attributable to interference with cell wall biosynthesis. However, if this were the only site of action, one would expect not only protoplast formation but actual L phase growth with typical colonies and large bodies visible after 4 to 7 days. This L growth would be such that it could be maintained in serial transfer.

It therefore follows that the antimicrobial agents investigated, namely, bacitracin, novobiocin, gentian violet and vancomycin cannot act solely by interfering with cell wall synthesis. They must exert additional actions elsewhere upon other metabolic or reproductive pathways.

*Summary.* The effect of bacitracin, novobiocin, gentian violet and vancomycin upon organisms known to be L forming bacteria was studied. L phase growth could not be produced although protoplast formation occurred in liquid media. It is concluded that these agents cannot act solely by interference with cell wall biosynthesis.

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## Chemical Protection Against Lethal Effects of Nitrogen Mustard in Rats.* (28754)

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Nitrogen mustard (HN2) is routinely dissolved in physiological saline solution before injection. Commercial saline, in its turn, often

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contains parabens as bacteriostatic agents; the effect of the parabens in conjunction with HN2 is unknown. The first series of experiments reported here was designed to test the effects of the propyl ester and the methyl ester of p-hydroxybenzoic acid (*propyl* and *methyl paraben*) on the action of HN2 in rats.

Although nitrogen mustard is carcinostatic, it is used reluctantly in cancer therapy because it has severe toxic side effects. Two classes of drugs were considered for use in conjunction with nitrogen mustard, in hopes that they could reduce its side effects.

1. Drugs that react with alkylating agents. (HN2 is an alkylating agent.) *Sodium tripolyphosphate* was selected because it was reported to react with alkylating agents *in vitro*(1) and is well tolerated by rats.

2. Drugs that reduce secondary infection. *Succinylsulfathiazole* was selected for oral administration because it could combat bacteremia resulting from damage to the intestinal mucosa by HN2(2).

*Materials and methods.* University of Rochester (Bronson Laboratory) and Carworth Farms adult, female, Wistar rats weighing 152-280 g were kept 3 to 4 in a cage on a diet of Purina Fox Chow, with or without additives, and with water *ad libitum*. Mortality was checked at 12 to 24 hour intervals for at least 14 and up to 30 days after HN2 treatment. Weights were taken before, and in some cases, after treatment. At least 10 rats were used to test each concentration of a drug, except for doses above 3.6 mg of HN2/kg, where 2 rats were used per dose per treatment (Fig. 2).

Nitrogen mustard (HN2) was prepared by dissolving mechlorethamine-HCl powder (Merck, Sharp & Dohme) in sterile physiological saline (0.9% sodium chloride) to a final concentration of 1 mg/ml. Injections were made (I.P.) within 20 minutes of drug preparation, and the order of administration of replicate doses was staggered. Doses were computed in mg of HN2 per kg of body weight.

Commercial physiological saline with paraben additive (Sodium Chloride Injection USP, Cutter Labs.) contained 50 mg of methyl paraben (methyl ester of p-hydroxybenzoic acid) and 5 mg of propyl paraben (propyl ester of p-hydroxybenzoic acid) per 100 ml solution. When nitrogen mustard was added, this "paraben saline" had 1 mol of paraben per 2 mols of HN2. Physiological saline with a higher concentration of paraben was prepared with 20 mg of methyl paraben and 2 mg of propyl paraben (USP, Heyden-Newport Chem. Corp.) in 10 ml of saline, to give 2 mols of paraben per mol of HN2. Saturated paraben saline was prepared by dissolving 10 mg of methyl and 1 mg of propyl paraben per ml of commercial saline with paraben additives at 80°C. Paraben diets were prepared with 20, 60, 180 and 540 mg of mixed methyl and propyl paraben (10:1) per 90 g of Fox Chow.

Sodium tripolyphosphate (anhydrous, Victor Chem. Corp.) was added to saline for injection at a concentration of 24.05 mg/10 ml, which was equimolar with HN2. Sodium tripolyphosphate diets were made up at 2% (w/w) in Purina Fox Chow, and were served *ad lib* to rats for 2 or 4 days prior to injection.

Rats were starved for 24 hours before succinylsulfathiazole (USP) administration. Three grams of drug per kg were then suspended in saline and administered by cannula to the stomach. After this preliminary forced feeding, the rats were placed on a 5% w/w diet.

Criteria used to determine the effectiveness of the drugs in reducing the toxic effects of HN2 were survival time, mortality rate, body weight changes and toxic signs (diarrhea, alteration of food and water uptake and changes in general appearance).

Numerical data were presented as means $\pm$ standard error.

*Results.* 1. *Calibration of drug effects.* There was no difference between the responses of University of Rochester rats and Carworth Farm rats to HN2 (Fig. 1). The $LD_{50}$ of HN2 for 150 rats weighing between 152 and 275 g was 1.90 $\pm$ 0.02 mg/kg (Fig. 1). Mean survival time for the rats that died from the nearest to $LD_{50}$ dose of 1.8 mg/kg was 5.4 $\pm$ 0.4 days.

Rats receiving from 1 to 5 $\times$ $LD_{50}$ of

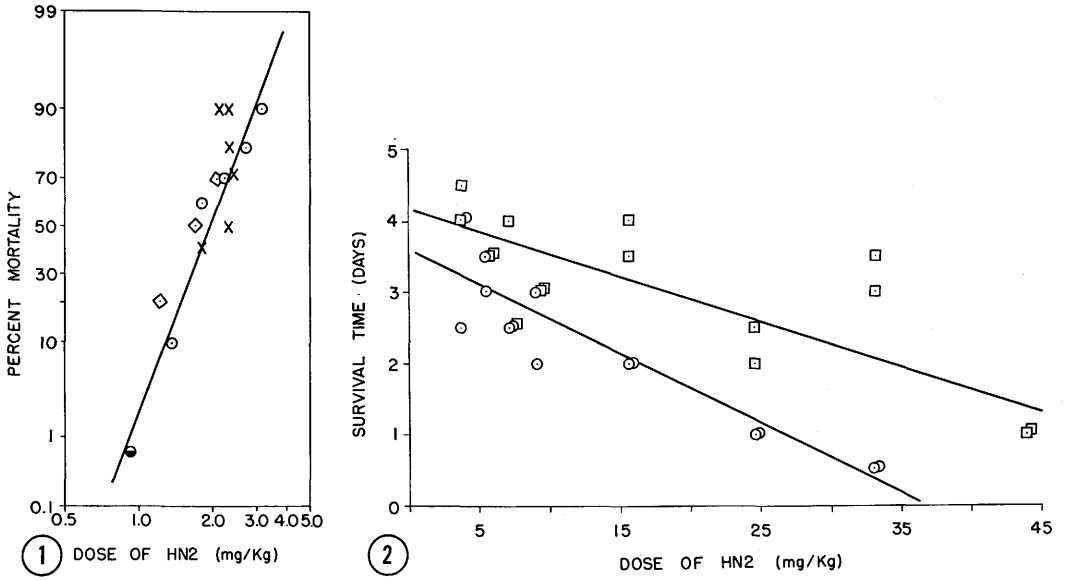


FIG. 1. Mortality of rats treated with HN2. Abscissa: dose of HN2 in mg/kg (intraperitoneal) on a logarithmic scale. Ordinate: percent of rats dead in 30 days plotted on a probit scale. Each point represents a group of 10 rats. □, A single experiment (3 groups of Carworth Farms origin). ○ and ◒, A single experiment (6 groups of Rochester origin). Shaded circle is a group with zero mortality plotted according to Litchfield and Wilcoxon(3). ×, Each group originates from a separate control experiment.

FIG. 2. Effect of paraben additive on survival time after supra-lethal doses of HN2. Abscissa: dose of HN2 in mg/kg of body weight. Dose of paraben was proportional to dose of HN2 and ranged from 1.97 to 6.03 mg/kg of mixed methyl and propyl paraben. Ordinate: survival time in days. Each point represents one animal. ○, HN2 in pure saline (controls). □, HN2 in paraben saline.

HN2, I.P. remained asymptomatic for 12 to 24 hours. A decrease in body weight started on the second day and was most severe on the 5th through 9th day, for survivors. On the second and third days the animals huddled in corners, their hair became ruffled, they neither ate nor drank, and they became progressively weaker. Watery diarrhea began about the third day and became severe by the fourth. Usually, if the rat survived 7 days recovery was rapid and complete. In a few scattered cases death occurred as late as 12 days post injection. No rats died between the 13th day and the end of the experiment (14 and 30 days). For doses below the $LD_{50}$, weight loss was delayed 1 or 2 days and was less severe.

Twelve rats tolerated the paraben diets for 5 days without change in weight, in general appearance, or in food consumption. Succinyl sulfathiazole (force fed, then a 5% diet) was equally well tolerated by 50 rats for 5 days. Prior experiments (Discussion) established the gut sterilization action of the succinylsulfathiazole diet and the tolerance of rats to a tripolyphosphate diet.

2. *Protection from supra-lethal doses of HN2.* Paraben added to the saline solution vehicle prolonged survival time of rats by as much as 1½ days, when the animals were exposed to acutely toxic (16-45 mg/kg) doses of HN2 (Fig. 2). In this dose range, death of control animals occurred prior to the third day after treatment, and before onset of diarrhea. Regression lines were fitted to the dose-mortality curves for the entire supra-lethal dose range of HN2 (3.6-43 mg/kg), and the slope of the line for paraben protected rats proved significantly different from the slope of the line for controls ($t = 0.05$). The lines converged in the lower doses, so that there was no significant protection by paraben from 9 mg/kg of HN2 on down (Fig. 2).

Tripolyphosphate gave no protection against supra-lethal doses of HN2. In the

TABLE I. Effects of Succinylsulfathiazole Diet on Survival of Rats Exposed to HN2 (2.3 mg/kg I.P.).

| Duration of diet | Other treatment | No. treated | No. dead | Mean survival time before death (days) |
|---|---|---|---|---|
| 5 days prior to HN2 | None | 20 | 7 | 8.0 |
| *Idem* and 7 days after | " | 10 | 7 | 8.3 |
| 5 days prior to HN2 | 5 mg/kg of methyl and propyl paraben (saturated solution) inj I.P. on second day after HN2 | 10 | 8 | 7.8 |
| *Idem* | HN2 inj in paraben-saline, and paraben inj I.P. (5 mg/kg) on second day after HN2 | 10 | 6 | 7.8 |
| None (control) | None | 50 | 38 | 4.1 |

dose range of 3.6 to 9.0 mg/kg of HN2, mean survival time for 8 rats injected with tripolyphosphate was 3.87 ± 0.28 days. In the dose range of 3.6 to 45 mg/kg of HN2, mean survival time for 16 rats on a tripolyphosphate diet was 2.0 ± 0.4 days. These readings were compared with the control reading of Fig. 1 (paired comparison test: 2 control animals and 2 experimental animals for each dose for each treatment) and were not significantly different.

3. *Protection from sub-lethal doses of HN2.* A. *Paraben.* Nine rats fed a 600 mg% diet of paraben for 12 days showed a significant increase ($t = .05$) in survival time, but not in mortality rate, after injection of 2.3 mg of HN2 per kg. Mortality was 80%, and mean survival time was 7.6 ± 0.7 days, as opposed to 4.1 ± 0.4 days for controls receiving the same dose of HN2. A shorter diet (600 mg%) fed to 10 rats for 8 days, or one with less paraben (22 mg%) fed to 13 rats for up to 22 days, altered neither survival time nor mortality rate.

Ninety rats were injected with one of the following doses of HN2 in paraben-saline: 1.7, 1.8, 2.3, 2.7, 3.15, 3.4, and 4.2 mg/kg. Thirty rats were given a prophylactic injection of 0.55 mg of paraben (I.M.) in solution, followed 3 hours later by injected doses of 2.7, 3.15, or 3.4 mg/kg of HN2. Ten rats received injections of 2.3 mg/kg of HN2 dissolved in a saturated solution of paraben in saline. Ten rats were injected with 2.3 mg/kg of HN2 in paraben-saline and given an additional 5 mg/kg of paraben on the second day. Finally, 10 rats were injected with 2.3 mg/kg of paraben-free HN2 and injected again with 5 mg/kg of paraben a day later. The mortality and life span after HN2 injection were the same for the 140 animals that received up to 2 mols of paraben per mol of HN2 as for the controls receiving HN2 only ($t = 0.05$).

B. *Succinylsulfathiazole.* The succinylsulfathiazole diet provided consistent protection against HN2 damage (Table I). The difference in survival time between experimental and control animals was statistically significant ($t = 0.05$). Mean survival time for experimental animals was 7.9 ± 0.9 days and that for controls was 4.1 ± 0.4 days. Survival was prolonged in every experiment; mortality was variable. In one experiment, 90% of the animals (9 out of 10) survived as compared with 30% of the controls. In other experiments succinylsulfathiazole did not decrease mortality. The protective action of succinylsulfathiazole was not enhanced by additional treatment with methyl and propyl paraben (Table I).

*Discussion.* HN2 given intraperitoneally to rats in this experiment had an $LD_{50}$ of 1.90 mg/kg ± 0.02. This is in good agreement with previous reports of $LD_{50}$'s for HN2 administered by various routes(4,5,6,7,8,9). Similarly, the toxic signs induced by HN2 and mean survival times of the exposed animals were within the range of those reported by others for HN2(4,5) and for hard radiation(10,11,12).

The greater the dose of X-rays the faster the death of the animal. This time difference is related to the organ system which is lethally injured. An $LD_{50}$ exposure caused death in 6 to 12 days and was ascribed to delayed

failure of the hematopoietic system(13,14, 15). If the dose was raised to 1.5 to 13 $\times$ $LD_{50}$, death occurred in 3 to 5 days after exposure and was caused by intestinal damage(13,15,16,17) which killed before the hematopoietic damage became apparent. A supra-lethal X-ray dose of 25 $\times$ $LD_{50}$ caused death within a day by damaging the central nervous system.

Rats reacted to HN2 in the same way as they did to X-rays with one important exception: they did not die because of a delayed failure of the hematopoeitic system. Those animals that died from a supra-lethal dose of HN2 did so within one day. Those that died from lower doses (6 $\times$ $LD_{50}$ and down) had a mean survival time of 4 days. This was true in the unprotected rats in our work, and in all cases reported in literature regardless of the method of administration of the drug. There was no third peak of mortality in the 6 to 12 day interval. Rats that died after HN2 exposure were killed either by central nervous system or intestinal tract damage and, unlike X-ray treated rats, they did not appear to die from delayed failure of the hematopoietic system after they recovered from the intestinal damage.

Treatments which protect against hard radiation will also protect against HN2(18). Four mechanisms of protection are known: (a) direct reaction with active radicals, (b) protective alteration of a target molecule, (c) antagonism of a toxic substance released by the recipient animal, and (d) supportive agents reversing the breakdown of some physiological function. Both the parabens and succinylsulfathiazole gave some protection against the toxic action of HN2. Parabens added to the saline prolonged the survival time of rats exposed to supra-lethal doses of HN2 (5 $\times$ $LD_{50}$ and up, Fig. 2). Survival time of rats exposed to lower doses of HN2 was not lengthened by paraben in saline, but was prolonged by a 600 mg% paraben diet. Paraben, in all probability, acted by mechanism (d) above. When mixed with HN2 *in vitro* paraben gave no protection against moderate doses of HN2 which indicated that it did not act by mechanism (b) or (c). Therefore, the paraben used for prolonged feeding probably acted as a general bacteriostatic agent much like succinylsulfathiazole, while the protective action of injected paraben against extreme doses of HN2 could be related to action on the central nervous system. It has been suggested that parabens are supportive to the CNS because of their pharmacologic relationship to the salicylates(19,20). In any case, the continued use of methyl and propyl paraben in therapeutic preparations of HN2 is quite justified in the light of our results.

Succinylsulfathiazole prolonged by 3 or 4 days the mean survival time of rats exposed to an $LD_{70}$ of HN2. The succinylsulfathiazole diet used in our experiments was designed to give a complete destruction of bacterial flora and sterility of the gut contents in rats(21). The sterility was confirmed experimentally in our strain of Wistar rats (E. Homan, unpublished). The sterilizing action is known to be beneficial in intestinal trauma (2) and consequently accounts for the protective action of succinylsulfathiazole in HN2 poisoning. When the protected rats died, they did so in 8 days on the average, which put them within the period of time ascribed to death from radiomimetic damage to the blood-forming system. It would appear that the intestine had to be protected before HN2 could kill rats by damaging the blood-forming system. These results suggest that systemically administered HN2 does more damage to intestinal lining than to the blood-forming tissues, while whole-body radiation does less.

Sodium tripolyphosphate ($Na_5P_3O_{10}$) reacts *in vitro* with alkylating agents and should, therefore, protect tissues from HN2 by mechanism (a) above(1). Neither prior feeding of sodium tripolyphosphate nor *in vitro* mixing with HN2 caused any inactivation of HN2 activity in the rat. A tripolyphosphate diet identical to the one used in this experiment was fed to rats in this laboratory for prolonged periods, and the animals showed no untoward effects (H. Hodge *et al,* pers. comm.): this eliminates the possibility that the toxic and beneficial actions of this

compound cancelled each other. Tripolyphosphate was not an antidote to HN2, either *in vitro* or in a target tissue, apparently because it resisted alkylation under physiological conditions.

*Summary.* 1. A dose-mortality curve for nitrogen mustard (HN2) given intraperitoneally to 150 Wistar rats gave an $LD_{50}$ of 1.9 $\pm$ S.E. $=$ 0.02 mg of HN2 per kg body weight. Mean survival times for groups of rats killed by moderate doses of HN2 ($LD_{10}$ through 2 $\times$ $LD_{50}$) were of the order of 4 days. Acute toxic doses (above 7 $\times$ $LD_{50}$) killed in 2 days or less. 2. Methyl and propyl paraben mixed into the saline solution used for injecting sub-lethal doses of HN2 had no effect upon either survival time or mortality of the rats. These additives apparently have no adverse effect on the action of HN2 in clinically acceptable doses. 3. A 600 mg% diet of parabens fed to rats receiving sublethal doses of HN2 increased survival time, but did not reduce mortality. Survival time of rats injected with acute toxic doses of HN2 was increased as much as 1½ days by parabens dissolved in the saline vehicle, with no change of mortality. 4. Pre-feeding of a 5% succinylsulfathiazole diet prolonged survival time of rats treated with HN2 consistently and significantly. 5. Tripolyphosphate administered by various routes had no protective effect upon survival time or mortality rate of rats injected with HN2. 6. The mechanism of action of the drugs is discussed.

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