Biochemical Pharmacology, Vol. 17, pp. 989–1002. Pergamon Press. 1968. Printed in Great Britain

INFLUENCE OF THE INITIAL pH VALUE OF SOLUTION ON THE ANTITUMOR ACTIVITY AND REACTIONS OF ALIPHATIC NITROGEN MUSTARDS*

GEORGE S. TARNOWSKI, FRANZ A. SCHMID, HARRY G. SATTERWHITE, EDWARD M. SOLNEY and RALPH K. BARCLAY

Division of Experimental Chemotherapy, Sloan-Kettering Institute and Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University Medical College, New York, N.Y., U.S.A.

(*Received* 15 *May* 1967; *accepted* 19 *December* 1967)

Abstract—Single i.p. doses of 3 mg/kg nitrogen mustard (HN2, diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride), administered to mice bearing 1-day-old Ehrlich ascites tumor (EA), led to a greater prolongation of median survival time of hosts and increased to a larger extent the number of survivors after 30 days or more when HN_2 was dissolved in vehicles with initial pH values of 5·6, 7·5 or 10·5 than with other initial pH values. Toxic doses of 6 mg/kg were better tolerated and prolonged survival time to a greater extent when administered in vehicles with the initial pH values of 2 or 4.

Single doses of 0·125–0·5 mg/kg HN2 administered the day after inoculation of EA decreased the total packed tumor cell volume measured on the eighth day of tumor growth to the greatest extent when the pH of solution at the time of injection was from 5·6 to 8·7. The pH-dependence of antitumor effect was less pronounced when the chemical was injected into mice on the fifth day of tumor growth. The pH of solutions at the time of injection influenced the antitumor activity of ethyl nitrogen mustard (triethylamine,2,2′-dichloro-) but not of nor-HN2 (diethylamine,2,2′-dichloro-,hydrochloride) or of chlorimine picrylsulfonate [1-(2-chloroethyl-)-1-methylaziridinium, 2,4,6-trinitrobenzenesulfonate]. The nature of the buffering substance influenced the antitumor activity in a few instances; phthalate, phosphate and Tris diminished the effects of nitrogen mustard and, to a greater extent those of ethyl nitrogen mustard.

The optimal pH range of the reaction *in vitro* of HN2 with thiosulfate was 6·3 to 9·5. The reaction of HN2 with 4-(*p*-nitrobenzyl) pyridine (NBP) was the most intensive at the initial pH value of 8. Aging of HN2 solutions with initial pH values of 5·5 or higher decreased its ability to react with either thiosulfate or NBP. The reactivity of HN2 with NBP showed no pH optimum when solutions of HN2 with different initial pH values were either mixed *in vitro* with cell-free ascites tumor fluid of the mouse, or when recovered from the peritoneal cavities of tumor-free mice into which HN2 solutions had been previously injected i.p.

THE pH VALUE at the time of injection of solutions of HN2 into tumor-bearing animals markedly influences the magnitude of antitumor activity and host toxicity, especially when administered in high doses. White[1] and White and Claflin[2] observed that the survival time of mice bearing Ehrlich ascites (EA) tumor or sarcoma 180 was greater when mice were treated with larger doses of HN2 at pH 2 than at pH 9. On the other hand, Cutts and Walker[3] found that "by a proper choice of dose, a pH 8 solution of HN2 can be just as effective or more so in its antitumor activity when compared

* This study has been supported by Grant CA-08748 from the National Cancer Institute.

with a pH 2 solution of HN2." In view of this discrepancy the antitumor activity of solutions of HN2 and other aliphatic nitrogen mustards with different pH values at the time of injection was investigated.

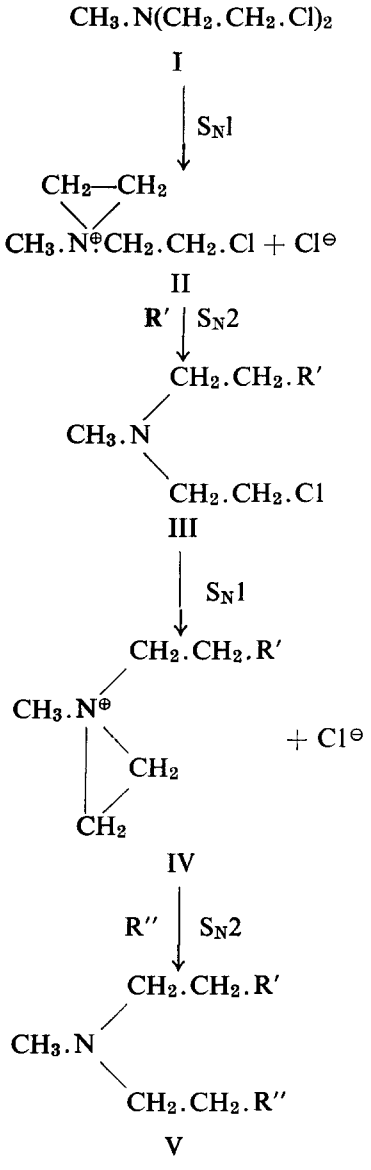

FIG. 1. Reactions of HN2 in aqueous solutions. R′, R″ = nucleophilic reactants.

Nitrogen mustards dissolved in aqueous media dissociate halogen ions first from one of the β-chloroethyl groups and then from the second (Fig. 1). The reaction in aliphatic mustards is first-order (S_N1) and its half-time at physiological pH and temperature is of the order of 1/2–2 min for the first chloroethyl group. It is slower for the

second chloroethyl group.[4-7] Dissociation of halogen ions leads to the formation of stable aziriiniumd (immonium) ions, which in turn react in a second-order (S_N2) reaction with nucleophilic groupings present in the medium. This reaction is thought to be responsible for the cytotoxic effects of nitrogen mustards.

The pH of the medium affects the rate of the formation of aziridinium ions and of their reactions with nucleophilic groupings of the medium. In the media with a pH lower than the pK_a of the protonation of the nitrogen atom of a mustard, the quaternary form prevails in the solution so that the rate of dissociation of halogen ions is decreased in the acid media.[8, 9] In many nucleophilic reactants, which contain hydrogen atoms ionizable in aqueous solutions, only one of the forms (protonated or non-protonated) will undergo alkylation by aziridinium ions and the concentration of the reactive form depends on the pH of the medium.

For these reasons, the pH-dependence of reactions *in vitro* of HN2 was examined with such nucleophilic reactants as thiosulfate ions and 4-(*p*-nitrobenzyl)pyridine-(NBP).

MATERIALS AND METHODS

EA was grown in ICR/Ha female mice, weighing 17–22 g at the time of i.p. inoculation of 10^7 tumor cells per mouse. Treatment with a nitrogen mustard started the day after inoculation, or 5 days thereafter. A single i.p. injection of a 0·5-ml volume of solution was administered, unless otherwise indicated, within 1–2 min after preparation. Each dose level was given to 5 mice; the control groups were treated with the vehicle in which the chemical was dissolved.

Effect of therapy was evaluated either by the median survival time of the treated hosts and the number of hosts surviving 30 days (or longer) or by the magnitude of the total packed cell volume (TPCV) on the eighth day of tumor growth. To determine the latter index of tumor growth, the mice were killed, the abdominal cavities were opened and a microcapillary glass tube was filled with ascitic fluid. After centrifugation at 10,000 rpm for 7 min in an International Hematocrit centrifuge, the fraction of the sample volume occupied by packed cells (F_c) was determined by means of the Cytocrit microcapillary reader. The abdominal cavity was then drained of ascites, dried with absorbent paper and the mouse was reweighed. The ascites volume (V_a) was calculated as the difference between the body weight with and without ascites. The density of ascitic fluid was assumed to be 1. The formula for computation was: TPCV (in ml) $= F_a \times V_a$.

Toxic effects of chemicals were estimated by the change of the average weight of treated mice. The averages were calculated on the basis of individual values of survival time, TPCV or weight change. All experiments were repeated at least 3 times.

Reaction of HN2 with thiosulfate ions.[10, 11] In different aqueous media, 10^{-2} M solutions of HN2 were aged at room temperature for 0, 2 and 24 hr. Four-ml aliquots were mixed with 2 ml of 0·1 N sodium thiosulfate and allowed to stand at room temperature for 2 hr. The solutions were then acidified with acetic acid; 200 mg solid potassium iodide was added and the thiosulfate ions, which did not react with HN_2, were titrated with 0·1 M iodine. The theoretical maximum amount of aziridinium ions present in samples of HN2 was 8×10^{-5} equivalents. The amount of thiosulfate combined with the aziridinium ions was determined as the difference between the added thiosulfate and the thiosulfate remaining after reaction with aziridinium ions.

Reaction of HN2 with NBP.[12] HN2 was dissolved in aqueous media with different initial pH values to make a $3{\cdot}4 \times 10^{-5}$ M solution. After incubation for 0–180 min at 37°, 1 ml of 0·025 M sodium acetate buffer, pH 4·6, was added to a 2-ml aliquot of the reaction mixture. The pH of the solution was adjusted to 4·6 with HCl or NaOH and the volume was brought to 10 ml with acetate buffer. One ml of 5% NBP in methylethylketone was added to 3-ml aliquots of buffered solution of HN2. The mixture was incubated at 37° for 20 min. Subsequently, 4 ml of 50% triethylamine in acetone was added and the volume was brought up with water to 10 ml. The absorbance was measured at 600 mμ in a model B Beckman spectrophotometer.

Reaction of HN2 with NBP in cell-free ascitic fluid.[13, 14] HN2 was dissolved in aqueous media with different initial pH values to make a $4{\cdot}17 \times 10^{-3}$ M solution, which was then diluted 1 : 8 with cell-free Ehrlich ascitic fluid. The latter was obtained by centrifuging ascites from mice bearing 6 to 7-day-old EA. After an incubation of 0–180 min at 37°, the mixtures were deproteinized by diluting 1:10 with 95% ethanol. Two-ml aliquots of deproteinized solutions of HN2 were brought to pH 4·6 by adding 2 ml of 0·025 M sodium acetate; 1 ml of 5% NBP in methylethylketone was then added and the mixture was incubated for 20 min at 37°. After cooling, 4 ml of 50% triethylamine in acetone and 1 ml of 1 M potassium carbonate were added. Absorbance readings were made at 565 mμ.

Materials. HN2 (as the hydrochloride) and chlorimine picrylsulfonate [1-(2-chloroethyl-)-1-methylaziridinium,2,4,6-trinitrobenzenesulfonate] were kindly supplied by Merck, Sharp & Dohme, Inc., and nor-HN2 was purchased from Beacon Chemical Co.

The solutions of mustards were prepared in the following solutions (pH values at 24°): hydrochloric acid, 0·01 N, pH 2; sodium acetate, 0·05 M, pH 4; sodium phthalate, 0·05 M, pH 5 and 5·5; saline, pH 5·6 (at room temperature, in air); sodium phosphate, 0·05, and 0·1 M, pH 6·5 and 7·5; imidazole, 0·05 M, pH 7·5; saline alkalinized with sodium bicarbonate to an initial pH of 8 in air; Tris (hydroxymethyl)aminomethane hydrochloride, 0·05 M, pH 8·5; sodium bicarbonate–sodium carbonate, 0·05 M, pH 8·5, 9·5 and 10·5.

Dissolution of HN2 in these solvents to make a $3{\cdot}4 \times 10^{-5}$ M solution did not alter the pH except in 3 instances. In saline the pH of HN2 solution decreased to 5·2 after 2 hr at room temperature and to 4·9 after 24 hr. In the bicarbonate-alkalinized saline the pH increased to 8·8 after 2 hr and to 9·4 after 24 hr. The pH value of bicarbonate–carbonate, 0·05 M, was adjusted to 8·5 in air and after HN2 was dissolved it immediately increased to 8·7, became 8·9 after 2 hr and 9·3 after 24 hr at room temperature.

RESULTS

Antitumor activity of mustards. The median survival time of mice bearing EA was studied after single i.p. injections of solutions of HN2 having different initial pH values the day after the inoculation of tumor. The surviving animals after 30 days of observation were free of ascites tumor. In the absence of HN2, the vehicle itself had no effect on the median survival time of the number of survivors on day 30 (Fig. 2). The well tolerated single dose of 3 mg/kg of HN2 prolonged survival time more when injected in solutions with initial pH values of 5·6, 7·5 or 10·5 than with pH values of 2, 4 or 9·5. After the dose of 4·5 mg/kg, the survival time also showed a dependence on both the pH value and the nature of the solvent; the prolongation of survival time was pronounced in mice treated with HN2 dissolved in the vehicles with pH values of

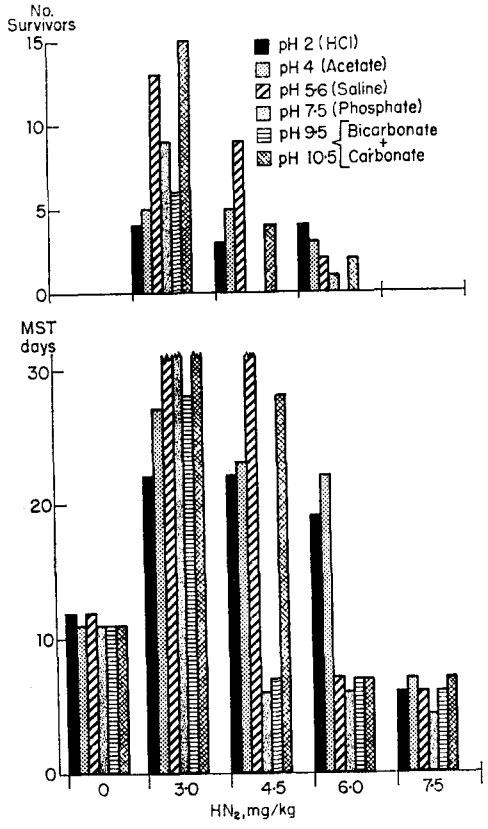

FIG. 2. Median survival time and the number of survivors at 30 days of mice (in groups of 15) bearing Ehrlich ascites tumor treated on the first day after implantation with single doses of 3·0–7·5 mg/kg HN2 in solutions having different initial pH values.

2, 4, 5·6 and 10·5, but not in those with pH values of 7·5 or 9·5. Only solutions with pH values of 2 and 4 prolonged the survival time of animals treated with single doses of 6 mg/kg HN2. The average weight loss 1 week after treatment with a single dose of 3 or 4·5 mg/kg HN2 was smaller when the chemical was dissolved in the acid medium (Fig. 3).

The TPCV of EA treated the day after implantation with a single dose of 0·125–0·5 mg/kg HN2, dissolved in vehicles with different pH values at the time of injection, was decreased the most when the initial pH was 5·6 (saline) to 8·7 (bicarbonate–carbonate) (Fig. 4). These low doses of HN2 had also markedly prolonged the survival time of tumor-bearing mice when administered in solutions with optimal initial pH values (Fig. 5). There were a few exceptions of diminished tumor inhibition: 0·125 mg/kg HN2 dissolved in 0·05 M Tris, pH 8·5; and 0·5 mg/kg HN2 administered in 0·05 M acetate, pH 4, or in 0·05 M phthalate, pH 5. Otherwise, no deviation from the general course of the pH-dependence of the antitumor effects of HN2 was observed which could be attributed to the nature of the buffering substance. Solvents administered alone did not affect the TPCV measured on the eighth day of EA growth.

The pH effects were less pronounced when single doses of HN2 dissolved in solvents with different initial pH values were administered i.p. on the fifth day after EA

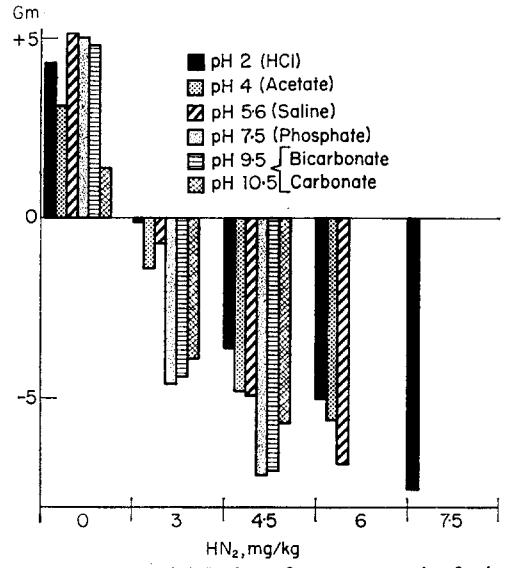

FIG. 3. Average weight change on the eighth day of tumor growth of mice bearing Ehrlich ascites tumor treated on the first day with single doses of HN2 in solutions having different initial pH values.

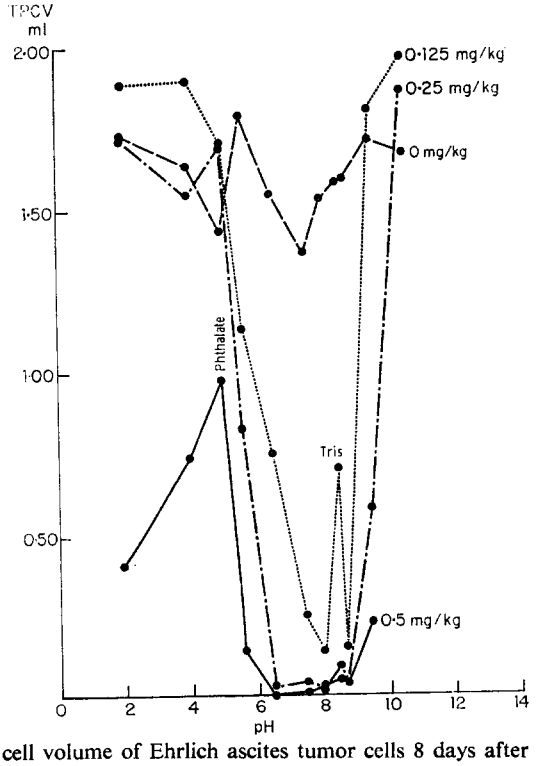

FIG. 4. Total packed cell volume of Ehrlich ascites tumor cells 8 days after implantation. Tumors had been treated on the first day with solutions of HN2 having different initial pH values. Solutions: HCl, pH 2; acetate, pH 4; phthalate, pH 5; saline, pH 5·6; phosphate, pH 6·5 and 7·5; bicarbonate–saline, pH 8; Tris, pH 8·5; bicarbonate–carbonate, pH 8·7, 9·5 and 10·5. Single i.p. doses of HN2. Pthalate and Tris decreased the antitumor activity of HN2.

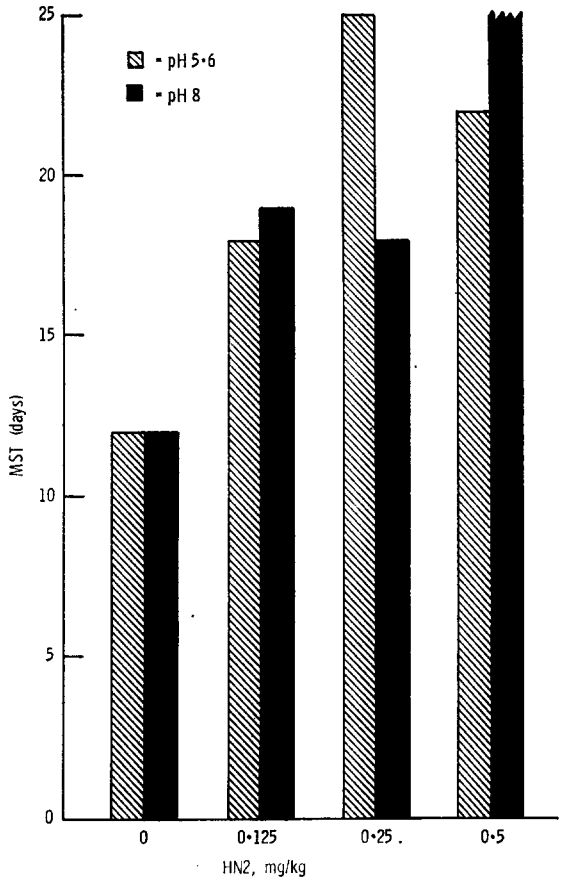

FIG. 5. Median survival time of mice (in groups of 10) bearing Ehrlich ascites tumor treated on the first day after implantation with single doses of 0·125–0·5 mg/kg HN2 in solutions having initial pH values of 5·6 (saline) and 8·7 (bicarbonate–saline).

implantation. The most pronounced decrease of TPCV measured on the eighth day of tumor growth occurred when HN2 was dissolved in bicarbonate–saline, pH 8 (Fig. 6). Doses of 1·5 or 3 mg/kg showed greater pH-dependence than the higher doses on the 5-day-old tumor.

Solutions of HN2 with initial pH values of 8·7 or 10·5, which were aged at room temperature for 2 or 24 hr, had diminished antitumor activity. Aging also affected, but to a lesser degree, the effectiveness of HN2 solutions prepared in saline. The activity of solutions in 0·01 N HCl was preserved.

The effect of the pH of solution at the time of injection was also studied in mice bearing 1-day-old EA treated with single i.p. doses of other aliphatic nitrogen mustards. Antitumor activity of ethyl nitrogen mustard showed marked dependence on the initial pH of its solutions and the nature of the buffering substance (Fig. 7). When administered as a single dose of 0·25–1 mg/kg the day after EA implantation, the most pronounced decrease of the TPCV on the eighth day of tumor growth was produced by solutions with pH 5·6–8·7 at the time of injection. The presence of phthalate,

phosphate or Tris ions in the medium markedly decreased tumor retardation especially at the dose of 0·25 mg/kg

No influence of the initial pH of solutions of nor-HN2 could be detected when it was administered as a single i.p. dose of 50–100 mg/kg; at these doses the TPCV was

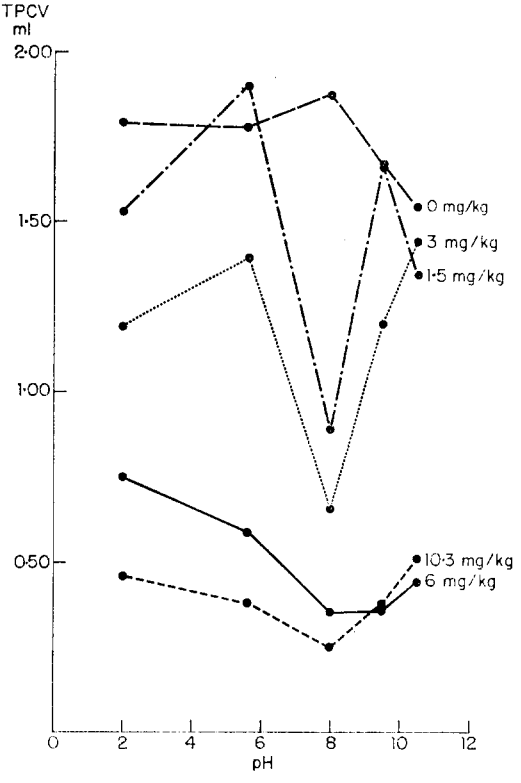

FIG. 6. Total packed cell volume of Ehrlich ascites tumor cells 8 days after implantation. Tumors had been treated on the fifth day with single i.p. doses of HN2 having different initial pH values. Solutions: HCl, pH 2; saline, pH 5·6; bicarbonate–saline, pH 8; bicarbonate–carbonate, pH 9·5 and 10·5.

decreased only slightly at any pH. However, at 200 mg/kg it was toxic, especially at higher pH values.

Chlorimine picrylsulfonate in single doses of 0·2–0·4 mg/kg decreased the TPCV of EA, but the effect was not influenced by the initial pH of solution below 10·5. At pH 10·5 the antitumor activity was greatly diminished.

Reactions of HN2 with nucleophilic reactants at different pH values. Freshly prepared solutions of HN2 with different initial pH values were incubated for 2 hr at room temperature in the presence of an excess of thiosulfate ions. The alkylated fraction of thiosulfate was negligible at pH 2 and 4; it increased between the pH values of 5 and 7 and then remained virtually constant between pH 7·5 and 10·5. In this pH range, 4×10^{-5} mole HN2, which when completely converted to aziridinium ions should yield 8×10^{-5} equivalents of alkylating species, reacted with 7·1 to $7{\cdot}4 \times 10^{-5}$ equivalents of thiosulfate ions (Fig. 8). This indicated that the preparation of HN2 used in the present study was at least 92·5 per cent pure.

When solutions of HN2 were aged at room temperature, the ability of solutions with initial pH values of 6·5 or higher to combine with thiosulfate was decreased after 2 hr and even more after 24 hr. Aging affected the reactivity of more acid solutions (phthalate, pH 5; saline, pH 5·6) to a lesser degree. At pH 2 or 4 the reactivity of HN2 with thiosulfate was not decreased by aging for 24 hr.

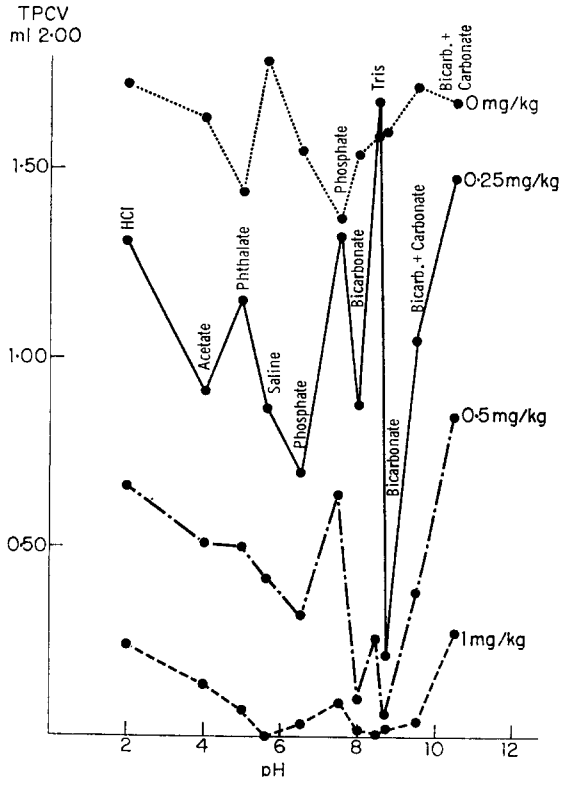

FIG. 7. Total packed volume of Ehrlich ascites tumor cells 8 days after implantation. Tumors had been treated on the first day with single doses of ethyl nitrogen mustard in solutions having different initial pH values. Solutions are the same as in Fig. 4.

The concentration of aziridinium ions in solutions of HN2 with initial pH values of 2–10·5 was determined by the colorimetric reaction with NBP. Solutions were aged for 0–180 min at 37°. The pH values were then adjusted to 4·6 with a 0·025 M sodium acetate buffer. After the reaction with NBP, the absorbance at 600 mμ was measured. In HN2 solutions aged for 0–60 min the concentration of aziridinium ions was the highest in solutions with the initial pH value of 8 (Fig. 9). The concentration of aziridinium ions decreased rapidly upon standing when the initial pH was 8 or 10·5. At initial pH values of 2 or 5·6 the absorbance values of the reaction product of NBP with freshly prepared HN2 solutions were lower than in the solutions with initial pH values of 8, but the decline of the ability to react with NBP upon aging was much less.

The pH-dependence of the concentration of aziridinium ions in the mixtures of freshly prepared solutions of HN2 having pH values of 2–10·5 with the cell-free fluid

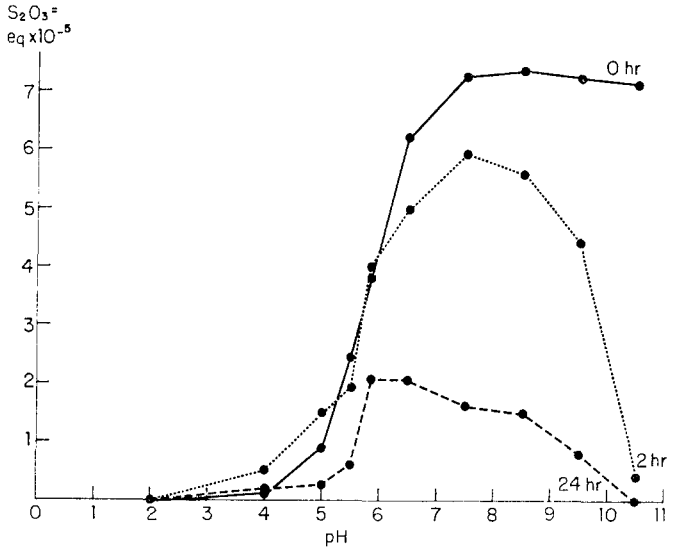

FIG. 8. Amount of thiosulfate ions (in equivalents $\times$ 10^{-5}) combined with aziridinium ions, derived from 4 $\times$ 10^{-5} mole HN2, after a 2-hr incubation at room temperature. HN2 solutions were aged 0–24 hr prior to incubation with thiosulfate. Solutions: HCl, pH 2; acetate, pH 4; phthalate, pH 5; saline, pH 5·6; phosphate, pH 6·5; imidazole, pH 7·5; bicarbonate–saline, pH 8; bicarbonate–carbonate, pH 8·7, 9·5 and 10·5. All solutions except HCl and saline were 0·05 M.

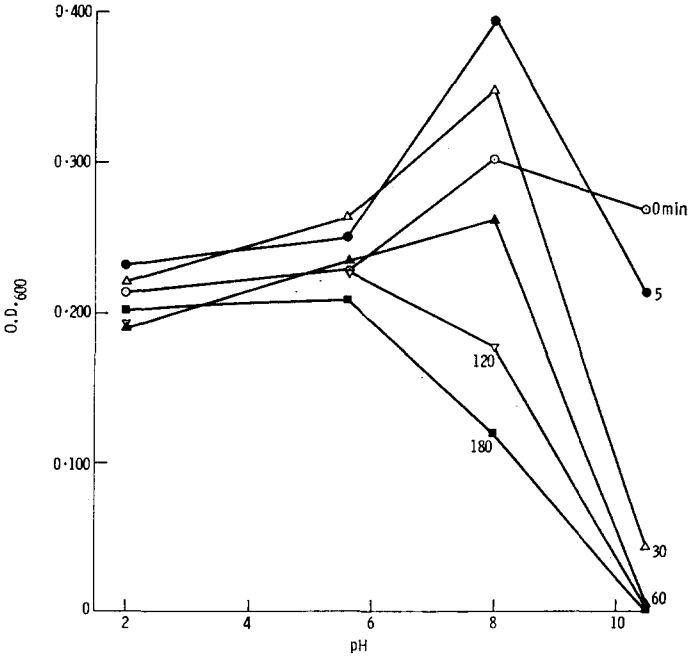

FIG. 9. Absorbance at 600 mμ of solutions of HN2 aged at 37° for 0–180 min and reacted with NBP after the adjustment of pH to 4·6. Solutions: HCl, pH 2; saline, pH 5·6; bicarbonate–saline, pH 8; bicarbonate–carbonate, pH 10·5.

of EA was studied. Aliquots of these mixtures incubated for 0–180 min at 37° were deproteinized with ethanol by following the procedures described by Klatt *et al.*[13] and Linford.[14] The concentration of aziridinium ions was determined by Klatt's modification of the NBP method. As the initial pH value of the HN2 solutions increased, the absorbance values in the deproteinized mixtures decreased. The decline was observable at all time intervals.

There was no pH-dependence of the concentration of aziridinium ions in the $3{\cdot}1 \times 10^{-4}$ M solutions of HN2 in aqueous media with different initial pH values, which were injected i.p. into tumor-free Swiss albino mice in a volume of 5 ml per mouse. Peritoneal fluid recovered after 0–180 min was pooled and deproteinized with 5% perchloric acid. The excess of perchloric acid was removed with KOH, the pH was adjusted to 4·6 with 0·05 M sodium acetate buffer, and the concentration of aziridinium ions was measured by using the reaction with NBP. The concentration of agaridinium ions in the peritoneal fluid declined markedly within 15 min after the injection of HN2 solutions. The rate of decline was independent of the initial pH value of the HN2 solutions. The disappearance of aziridinium ions from the peritoneal cavities continued at the later time intervals at a diminished rate; after 180 min virtually no aziridinium ions remained.

DISCUSSION

The initial pH value of solutions of HN2 influenced the survival time of treated mice bearing EA at the high single dose of 6 mg/kg. The median survival time was higher in animals treated with HN2 dissolved in acid media (initial pH 2 or 4) than in more alkaline solutions. The well tolerated single dose of 3 mg/kg prolonged the survival time the most when the solutions had initial pH values of 5·6, 7·5 and 10·5. These observations partially reconcile the discrepancy between the findings of White and Claflin[2] and those of Cutts and Walker.[3] The data are not strictly comparable in respect to the effects observed in EA because White and Claflin used single doses of HN2 varying from 2·4 to 6·5 mg/kg for the treatment of 1-day-old tumors, whereas Cutts and Walker treated 3-day-old EA with 5 doses of 0·2 mg/kg/day administered once a day. This latter, well tolerated, repeated dose prolonged the survival time longer when it was administered in a pH 8 solution rather than in HCl–saline at pH 2. This outcome is comparable to our findings with the single dose of 3 mg/kg. On the other hand, single, more toxic doses of 4·5 or 6 mg/kg administered in a pH 2 solution, as used by White and Claflin, caused a much greater prolongation of survival time than 4·5 mg/kg at pH 5·1 or 3·5 mg/kg at pH 9·3. This observation corresponds to our observation that a single dose of 6 mg/kg HN2 was more effective and less toxic when injected in an acid solution.

Survival time as a measure of antitumor activity and body weight loss as a measure of host toxicity are believed to be appropriate indices for estimating the anticipated clinical performance of an antitumor drug. To study the mechanisms of antitumor action of a chemical, an index such as TPCV of an ascites tumor is a superior index because it better evaluates the effect of treatment on the rate of proliferation of tumor cells *in vivo*. When applied to 1-day-old EA treated with single doses of HN2 in solutions with different initial pH values, the TPCV measured 6 days after treatment indicated that the tumor-retarding effects were the greatest when the initial pH was 5·6–8·7. The decline of antitumor activity of HN2 in solutions with a pH lower than

5·6 is attributable to the increase of the concentration of the protonated, quaternary form of HN2 in more acid solutions, since the pK_a of the protonation of the nitrogen atom at 25° is 6·55.[15] The protonated form of HN2 does not release halogen ions in solution and does not form the cytotoxic aziridinium ions. The decreased effect of alkaline (pH 9·5 and 10·5) solutions of HN2 on the TPCV is caused by the increased competition of hydroxyl ions of the solvent for aziridinium ions.

This competition of nucleophilic reactants for aziridinium ions explains many observations made in the present study. In 1-day-old tumors the peritoneal cavities of the mice contained little fluid and not more than 2×10^7 tumor cells (assuming that the cells of an inoculum of 10^7 cells have all duplicated in 24 hr). Even small variations in concentration of aziridinium ions in the injected solutions of HN2 would result then in markedly different antitumor effects. The peritoneal cavities of mice bearing 5-day-old EA contain 3–4 ml of ascitic fluid with approximately $1–1·5 \times 10^8$ cells per ml, and a concentration of proteins similar to that in the blood plasma. The numerous tumor cells and protein molecules of the ascitic fluid enhance its buffering capacity, compete for the injected aziridinium ions, and mask the effects of the differences in concentration of these ions in injected solutions of HN2.

With three exceptions, the nature of the buffering substance did not distort the general course of the plot of the TPCV of EA against the initial pH value of solutions of HN2 (Fig. 4). On the other hand, the presence of phthalate, phosphate or Tris ions in the solvent markedly altered the nature of the pH-dependence of antitumor effects of ethyl nitrogen mustard at lower doses (Fig. 6). This suggests that the buffering ions play a greater role of competition for the aziridinium ions of this mustard.

The antitumor activity of chlorimine picrylsulfonate and nor-HN2 was independent of the pH value of the medium. Chlorimine[11] is the product of the first, rapid and pH-dependent reaction of HN2 in aqueous media (substance II, Fig. 1) and, since the ionization and cyclization of the second chloroethyl group is slow, the concentration of the cytotoxic molecular species in its solutions is not affected by the pH of the medium lower than 10·5. At the latter pH value the hydrolysis of chlorimine to less cytotoxic molecular species diminishes the antitumor activity of solutions of this chemical.

Nor-HN2 is a secondary amine. Its reactions in aqueous solutions are different from those of other aliphatic nitrogen mustards, which are tertiary amines at pH values higher than the pK_a of the protonation of their nitrogen atom. Cyclization of nor-HN2 leads to a release of a proton and the formation of a cyclic imine. The reactivity of cyclic imine is lower than that of aziridinium ion and not dependent on the pH value of the solution. Therefore, the relatively weak antitumor activity of nor-HN2 is not dependent on the initial pH value of its solution.

The formation of aziridinium ions from nitrogen mustards in aqueous solutions is such a rapid reaction at room temperature that the equilibrium concentration of the aziridinium ions is achieved by the time a freshly prepared solution of a mustard is injected i.p. The reaction of aziridinium ions with the nucleophilic reactants of the solvents is much slower, and the aziridinium ions are relatively long-lived. These phenomena explain the dependence of antitumor effects of mustards on the initial pH value of their solutions, even though their pH values almost instantaneously changed to between 7 and 8 after injection into the peritoneal cavity (White and Claflin).[2]

With respect to dependence on the initial pH value of the HN2 solution, two types of reactions *in vitro* of HN2 with nucleophilic reactants were observed in the present study. Aziridinium ions formed from HN2 combine with thiosulfate and other present nucleophilic species, including OH^- (Fig. 1). Aging of solutions of HN2 allows aziridinium ions, hydroxyl ions and other nucleophilic species of the buffer solutions to react to such an extent that a smaller number of unreacted aziridinium ions remains available to react with thiosulfate ions. The fraction of thiosulfate combined with aziridinium ions markedly declines after a 2-hr aging of HN2 solutions with initial pH values of 9·5 and higher. At pH 5·6–8·7 the decrease of the alkylation of thiosulfate is much smaller because the concentration of hydroxyl ions in solution is lower. In freshly prepared solutions of HN2 the competition of hydroxyl with thiosulfate ions for aziridinium ions is negligible because of the great disparity in magnitude of the competition factors for the two nucleophilic reactants.[16]

In the case of the NBP reaction, the color formation depends on the concentration of aziridinium ions present when a solution of HN2 is mixed with a solution of NBP. Fig. 9 shows that the concentration of aziridinium ions was higher in freshly prepared solutions of HN2 with initial pH values of 8 and 10·5 than 2 or 5·6. This result corresponds to the findings obtained with freshly prepared solutions of HN2 in reaction with thiosulfate. The disappearance of aziridinium ions upon aging was rapid at pH 10·5, slower at pH 8, and negligible at pH 5·6 and pH 2. This was the expected result of the reaction of aziridinium ions with hydroxyl ions in more alkaline solutions.

The growth-retarding activity of nitrogen mustards evidently results from a series of interlocking consecutive reactions of the administered chemical and of the derived molecular species during the course of such reactions. Aziridinium ions (such as ions II, Fig. 1 in the case of HN2) are the principal molecular species which react with nucleophilic reactants in solutions *in vitro* and biologically after administration to a tumor-bearing host. The concentration of these ions in the injected solutions of HN2 depends on the initial pH, the chemical composition and the age of solution. Whereas in a mouse bearing an ascites tumor the pH of the injected solution is almost instantaneously changed because of buffering with the contents of the peritoneal cavity, the pH-dependence of the effects of the administered mustard on the TPCV appears to reflect the concentration of aziridinium ions present in the solutions at the time of injection.

REFERENCES

1. L. P. White, *Proc. Am. Ass. Cancer Res.* **3**, 162 (1960).
2. L. P. White and E. F. Claflin, *J. Pharmac. exp. Ther.* **142**, 265 (1963).
3. J. H. Cutts and I. G. Walker, *Cancer Res.* **26**, 1386 (1966).
4. F. S. Philips and A. Gilman, in *Approaches to Tumor Chemotherapy* (Ed. F. R. Moulton), p. 285. Am. Ass. Advancement of Sciences, Washington (1947).
5. W. C. J. Ross, *Adv. Cancer Res.* **1**, 397 (1953).
6. C. C. Price, *Ann. N.Y. Acad. Sci.* **68**, 663 (1958).
7. G. P. Wheeler, *Cancer Res.* **22**, 651 (1962).
8. W. C. J. Ross, *Biological Alkylating Agents*, pp. 11 and 29. Butterworths, London (1962).
9. O. M. Friedman and E. Boger, *Analyt. Chem.* **33**, 906 (1961).
10. C. Golumbic, J. S. Fruton and M. Bergmann, *J. org. Chem.* **11**, 518 (1946).
11. C. C. Hunt and F. S. Philips, *J. Pharmac. exp. Ther.* **95**, 131 (1949).
12. J. Epstein, R. W. Rosenthal and R. J. Ess, *Analyt. Chem.* **27**, 1435 (1955).

13. O. KLATT, A. C. GRIFFIN and J. S. STEHLIN, JR., *Proc. Soc. exp. Biol. Med.* **104**, 629 (1960).
14. J. H. LINFORD, *Biochem. Pharmac.* **8**, 343 (1961).
15. N. BROCK and H. J. HOHORST, *Arzneimittel-Forsch.* **11**, 164 (1961).
16. A. G. OGSTON, *Trans. Faraday Soc.* **44**, 45 (1948).