[CANCER RESEARCH 49, 2965–2972, June 1, 1989]

Hydrogen Ion-mediated Enhancement of Cytotoxicity of Bis-Chloroethylating Drugs in Rat Mammary Carcinoma Cells *in Vitro*[1]

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ABSTRACT

Aerobic glycolysis, a metabolic characteristic of malignant cells, can be exploited to increase the concentration of lactic acid selectively in tumor tissues *in vivo* by systemic administration of glucose (E. Jähde and M. F. Rajewsky, Cancer Res., *42:* 1505–1512, 1982). To investigate whether a more acidic microenvironment can enhance the effectiveness of cytocidal drugs, we have analyzed the colony-forming capacity of M1R rat mammary carcinoma cells exposed to bis-chloroethylating agents in culture as a function of extracellular pH (pH_e). At pH_e 6.2 the cytotoxicity of 4-hydroperoxycyclophosphamide, as measured by inhibition of colony formation, was potentiated by a factor of ~200 as compared to pH_e 7.4. Similar results were obtained with mafosfamide, nitrogen mustard, nor-nitrogen mustard, melphalan, and chlorambucil; not, however, with ifosfamide. As indicated by experiments using the ionophor nigericin for rapid equilibration of pH_e and intracellular pH (pH_i; measured with pH-sensitive microelectrodes), modulation of drug action by varying pH_e primarily resulted from the concomitant decrease in pH_i. The acidic microenvironment enhanced cytotoxicity most effectively during the phase of cellular drug uptake and monofunctional alkylation of DNA. DNA cross-link formation appeared to be less affected by pH, and lowering of pH_e during the phase of cross-link removal was only marginally effective.

INTRODUCTION

The therapeutic efficiency of anticancer drugs is severely limited by their toxic side effects on critical normal cell systems. The excellent therapeutic index of bactericidal as opposed to cytocidal chemotherapeutic agents is based on the exploitation of metabolic differences between bacteria and mammalian cells. In designing cytocidal drugs and strategies for cancer treatment, few investigators (1, 2) have thus far exploited aerobic glycolysis, a metabolic property distinguishing most malignant cells, of both animal and human origin, from their normal counterparts (3, 4; Table 1; for exceptions see Ref. 5).

The amount of lactic acid formed by malignant cells is highly sensitive to changes in the supply of glucose (6, 7). The glucose concentration in the interstitial fluid of malignant tumors is very low in comparison to arterial blood or the interstitial fluid of normal tissues (8). Therefore, the glycolytic rate of cancer cells *in vivo* is generally far below its potential maximum (6, 7) and may be increased by an elevated supply of glucose. Since lactic acid concentration is closely correlated to the concentration of H^+ ions (9), an increased glycolytic rate of tumor cells leads to a reduction of intratumoral pH (9, 10). Various studies have shown that the intratumoral concentration of H^+ ions can indeed be increased up to 10-fold in primary and transplanted rodent tumors by systemic administration of glucose *in vivo* (mean pH, ~6.2; Table 1). When the glycolytic activity of transplanted TV1A rat neurinomas was increased by i.v. infusion of glucose, the mean intratumoral pH decreased from about 6.9 to 6.1 in a tumor-selective manner (10). pH distributions in normal tissues of the tumor-bearing hosts were only marginally affected by the hyperglycemia (Table 2). Similar results were obtained with other tumors of epithelial and mesenchymal origin (Table 1), and confirmed by pH measurements in human tumors (11, 45).

In the present study we have investigated whether the cytotoxic effects of bis-chloroethylating drugs on malignant cells in culture can be modified by varying pH_e[3] over the range previously measured in malignant tumors with and without glucose perfusion. Specifically, the following questions were addressed: (*a*) is the cytotoxicity of alkylating drugs, in particular of nitrogen mustard derivatives like CP and its analogs, dependent on microenvironmental pH; and (*b*) which molecular mechanisms may be sensitive to an H^+ ion-mediated modulation of drug cytotoxicity? Bis-chloroethylating agents were chosen for two reasons: It is known that ethyleneimines, a structurally different group of alkylating compounds, exhibit a higher cytotoxic activity at reduced pH, probably due to protonation of the nitrogen atoms of the ethyleneimmonium rings resulting in increased reactivity (12). Moreover, it has been suggested that chloroethylating agents like CP and its metabolites also react via formation of an immonium ion intermediate (13, 14), and a recent study has shown that the action of CP on malignant cells in culture may be dependent on pH_e (15). In the latter analyses mouse serum containing CP and its various metabolites in undefined quantities was used as the source of aCP. We have attempted to quantitate the influence of H^+ ion concentration on the cytotoxic action of CP and to evaluate the role of CP metabolites in pH-dependent cytotoxicity, using chemically defined preparations of aCP and its metabolites. In addition, the effect of an acidic environment on the cytotoxic action of IFO, an analog of CP, and of bis-chloroethylating agents structurally different from oxazaphosphorines was investigated.

MATERIALS AND METHODS

Drugs

4-OOH-CP, MAFO, and 4-OOH-IFO were kindly provided by Dr. P. Hilgard (Asta-Werke, Bielefeld, FRG). Drugs were stored at −20°C

Received 9/27/88; revised 2/17/89; accepted 2/24/89.

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[1] This investigation was supported by the Federal Ministry for Research and Technology, by BYK Gulden Fonds für Experimentelle Krebsforschung, by Grant 85.003.1 from the Wilhelm Sander-Stiftung, and by the Deutsche Forschungsgemeinschaft (Hu 204/9-4). Presented in preliminary form at the Annual Congress of the German Society of Hematology and Oncology, Tübingen, Federal Republic of Germany, October 5–8, 1986.

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[3] The abbreviations used are: pH_e, extracellular pH; pH_i, intracellular pH; aCP, "activated" cyclophosphamide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BIS-TRIS, 2-[bis(2-hydroxyethyl)imino]-2-(hydroxymethyl)-1,3-propanediol; CP, cyclophosphamide; DMEM, Dulbecco's modified Eagle's minimal essential medium; HN2, mechlorethamine; IFO, ifosfamide; L-PAM, melphalan (L-phenylalanine mustard); MAFO, mafosfamide; nor-HN2, nornitrogen mustard [bis-(2-chloroethyl)-amine-hydrochloride]; 4-OH-CP, 4-hydroxycyclophosphamide; 4-OOH-CP, 4-hydroperoxycyclophosphamide; 4-OOH-IFO, 4-hydroperoxyifosfamide; PBS, phosphate buffered saline; pH-DMF, pH-dose modifying factor.

Table 1 *pH in malignant tumors of normo- and hyperglycemic hosts*

Tumor	Technique for pH measurement	pH values (tumor tissue)		Ref.
		Normoglycemia	Hyperglycemia	
Malignant melanoma (human)	pH microelectrodes	6.9	6.5	(11)
Tumors of varying histology (human)	pH microelectrodes	7.1	6.9	(45)
DS-Carcinosarcoma (rat)	pH microelectrodes	7.0	6.4	(9)
DS-Carcinosarcoma (rat)	pH microelectrodes	6.9	6.1	(46)
Yoshida sarcoma (rat)	pH microelectrodes	7.2	6.6	(47)
TV1A neurinoma (rat)	pH microelectrodes	6.9	6.1	(10)
HV1A3 carcinoma (rat)	pH microelectrodes	6.9	6.1	(10)
DS-Carcinosarcoma (rat)	pH microelectrodes	6.8	6.2	(48)
Guerin carcinoma (rat)	pH microelectrodes	6.8	5.6	(41)
Primary skin tumors (rat/mouse)	pH microelectrodes	7.0	6.1	(44)
RIF-1 fibrosarcoma (mouse)	pH microelectrodes	7.1	6.9	(43)
Walker 256 carcinoma (rat)	TIF sampling[a]	7.0	6.0	(49)
RIF-1 fibrosarcoma (mouse)	^{31}P-NMR	7.0[b]	6.5[b]	(33)
Mean		6.97	6.29	

[a] Tumor interstitial fluid sampling.
[b] pH_i (not included in mean value).

Table 2 *pH in normal tissues of normo- and hyperglycemic tumor-bearing hosts*

Tissue	pH values		Ref.
	Normoglycemia	Hyperglycemia	
s.c. tissue (human)	7.4	7.4	(11)
s.c. tissue (human)	7.4	7.4	(45)
s.c. tissue (rat)	7.2	7.1	(49)
Skeletal muscle (rat)	7.4	7.3	(9)
Skeletal muscle (rat)	7.4	7.4	(46)
Liver (rat)	7.2	7.2	(47)
Brain (rat)	7.0	6.9	(10)
Kidney (rat)	7.1	7.0	(10)
Liver (mouse)	7.2	6.6	(43)
Skeletal muscle (mouse)	7.2	7.1	(43)
Mean	7.25	7.14	

and freshly dissolved in PBS (pH 5.0; 4°C) immediately prior to use. HN2 and L-PAM, respectively, were obtained as pharmaceutical preparations from Merck, Sharp & Dohme (Vienna, Austria) and Deutsche Wellcome (Burgwedel, FRG). HN2 was dissolved in H_2O immediately before use. Solutions of L-PAM were prepared according to the manufacturer's recommendations for i.v. application. Chlorambucil, a gift from Dr. J. Schumann (Deutsche Wellcome), was dissolved in isotonic saline. Nor-HN2 was purchased from Aldrich (Steinheim, FRG) and solutions were prepared using PBS (pH 5.0). Nigericin (Sigma, Deisenhofen, FRG) was dissolved and diluted in 50% ethanol (v/v) and aliquots of 50 µl were added to each culture dish. Care was taken not to exceed 10 min from dissolving the drugs until addition to the culture medium.

Cells and Culture Conditions

BICR-M1$R_{k\text{-}d}$ cells (16; for simplicity referred to as M1R cells throughout the text) were used in all experiments. This clone was derived from the BICR-M1R_k clonal cell line which originated from a spontaneous mammary carcinoma of a Marshall rat (16). Cells were grown in DMEM supplemented with 10% newborn calf serum, penicillin, and streptomycin, and kept in a humidified incubator at 37°C under an atmosphere of 93% air and 7% CO_2. Drug exposure was performed in modified DMEM containing BIS-TRIS (20 mmol/liter) and $NaHCO_3$ (15 mmol/liter). The pH of the culture medium was adjusted to the appropriate value by adding 0.1 N HCl or 0.1 N lactic acid and taking into account the pH shifts after gassing with CO_2. Drugs were added in aliquots of ≤50 µl. These volumes of drug containing solutions did not significantly change the pH of the culture medium (5 ml per dish). The pH of the dishes was controlled following addition of the drugs and at the end of drug exposure. Dishes differing from the appropriate value by more than 0.1 pH unit were discarded.

Drug Exposure and Plating

Log phase M1R cells were incubated with the drugs for 24 h unless otherwise stated (17). Thereafter, cells were rinsed twice with drug-free DMEM, pH 7.4, trypsinized and counted. 10^2, 10^3, 10^4, or 10^5 cells/dish, respectively, were plated in triplicate onto 60-mm Falcon plastic dishes containing DMEM (pH 7.4). After incubation for 12 days colonies were fixed, stained with Loeffler's methylene blue, and colonies with a diameter >1 mm (~1000 cells) were counted. As shown in separate experiments, there was a linear relationship between the number of cells plated and the number of colonies formed over a range of 25–500 cells per plate. The colony-forming fraction of cells surviving drug treatment was calculated as the ratio of the number of colonies formed to the number of drug-exposed cells inoculated. Calculated values were normalized to the plating efficiency of untreated control M1R cells and corrections were made for the fraction of cells lysed during drug exposure according to the following formula:

Colony-forming fraction

$$= \frac{\text{(plating efficiency) exposed}}{\text{(plating efficiency) control}} \times \frac{\text{(cells/dish) exposed}}{\text{(cells/dish) control}}$$

Results are presented as mean values of three to six separate experiments with the standard deviations (not shown), typically, being less than 30% of the means.

Measurements of Intracellular pH

Microelectrodes. Fiber-containing borosilicate capillaries (Hilgenberg, Malsfeld, FRG), 1.0-mm outer diameter and 0.5-mm inner diameter, were cleaned by immersion (24 h) in chromic sulfuric acid followed by repeated rinsing in distilled H_2O. Micropipets were pulled with a vertical puller (700C; David Kopf Instruments, Tujunga, CA). Tip resistance varied between 10–20 MΩ when the pipets were filled with 3 M KCl solution. Micropipets for H^+ ion-selective microelectrodes were dried at 180°C for 20 min. Their tips (diameter, ~0.5 µm) were silanized by dipping into a solution of 10% (v/v) trichloromethylsilane (Fluka, Buchs, Switzerland) in tetrachloromethane (Merck, Darmstadt, FRG) for 20 s and subsequently baked in an oven at 100°C for 30 min. Silanized micropipets could be stored over silica gel for several days without alterations of tip resistance. Micropipets were filled with a H^+-ligand cocktail (Fluka) based on tridodecylamine (18) with the use of a syringe and a fine glass drain tube. The top of the microelectrode shank was filled with the ion-selective liquid [height, ~3 mm], followed by backfilling with internal filling solution [40 mM KH_2PO_4-23 mM NaOH-15 mM NaCl buffered to pH 7 (18)]. Conventional microelectrodes for measuring membrane potentials were backfilled with 3 M KCl solution.

Calibration. H^+-sensitive microelectrodes were calibrated in BIS-TRIS buffered DMEM (pH 6.15–7.9) before and after experiments. The corresponding microelectrode potentials were plotted as a function of pH. The mean slope and resistance of the pH-sensitive microelectrodes were 58 ± 2.2 mV (mean ± SE; n = 12) and ~100 GΩ, respectively. Microelectrodes were equilibrated at room temperature and used at 37°C, as the ion-sensitive liquid was found to be insensitive to temperature changes over this range (19).

Electrical Measurements. Measurements of membrane potentials and pH_i were performed under a phase-contrast microscope (Epivert; Leitz, Wetzlar, FRG). The two recording microelectrodes were connected to the input of a high impedance amplifier (FD 223; WP Instruments,

New Haven, CT). The reference electrode in 3 M KCl solution was connected to the sample solution via a Ringer agar bridge. Potential differences were recorded with a pen recorder (S 600; Gebr. Laumann, Selb, FRG). All electrophysiological measurements were performed in BIS-TRIS buffered DMEM at 37° ± 1°C. pH was adjusted to the required value by titration with 1 M HCl or 1 M NaOH, respectively. Micromanipulators with electrical drive (Gebr. Märzhäuser, Wetzlar, FRG) ensured controlled microelectrode movements.

RESULTS

Colony-forming Capacity of M1R Cells at Reduced pH_e. The effect of an acidic microenvironment *per se* on the colony-forming capacity of M1R cells is shown in Fig. 1. The fractions of colony-forming cells decreased with decreasing pH_e and as a function of the duration of exposure to reduced pH_e, respectively. However, this effect became significant only below pH_e 6.3 and after exposure times ≧24 h. At pH_e 6.3 the fraction of colony-forming cells still amounted to 65% of control (pH_e 7.4) after 24-h exposure. Even at pH_e 5.9 an incubation >24 h was required to lower this value to <10%. In order to minimize the influence of reduced pH_e *per se* on the results of the following experiments, drug exposure was limited to 24 h, unless otherwise stated.

Cytotoxicity of aCP on M1R Cells as a Function of pH_e. CP is a prodrug requiring microsomal hydroxylation for the formation of its first reactive metabolite, 4-OH-CP. Since 4-OH-CP is highly unstable in aqueous solution, a number of self-activating CP derivatives were used instead. Among these were 4-OOH-CP, a stabilized form of 4-OH-CP, and MAFO, a new oxazaphosphorine compound (20, 21). Both derivatives undergo rapid spontaneous hydrolysis in H_2O, with liberation of 4-OH-CP (20, 21).

To determine whether a reduced pH_e influences the cytotoxicity of aCP, log phase M1R cells were incubated with 4-OOH-CP at different pH_e (Fig. 2). At pH_e 7.4 the fraction of colony-forming cells (4-OOH-CP concentration, 1.0 µg/ml) was ~8% of untreated controls; the corresponding value at pH_e 6.2 was ~0.04%, representing a 200-fold enhancement of aCP cytotoxicity at pH_e 6.2 as compared to pH_e 7.4. Cytotoxicity was further enhanced by lowering pH_e to 5.6. At this pH_e inhibition of colony-formation by aCP was potentiated by a factor of $\sim 10^3$ relative to control cells exposed to aCP at pH_e 7.4. The observed H^+ ion-mediated enhancement of aCP cytotoxicity was independent of the kind of prodrug applied. Essentially the same results were obtained when MAFO (2.5 µg/ml) was used as a precursor of 4-OH-CP instead of 4-OOH-CP (Fig. 3).

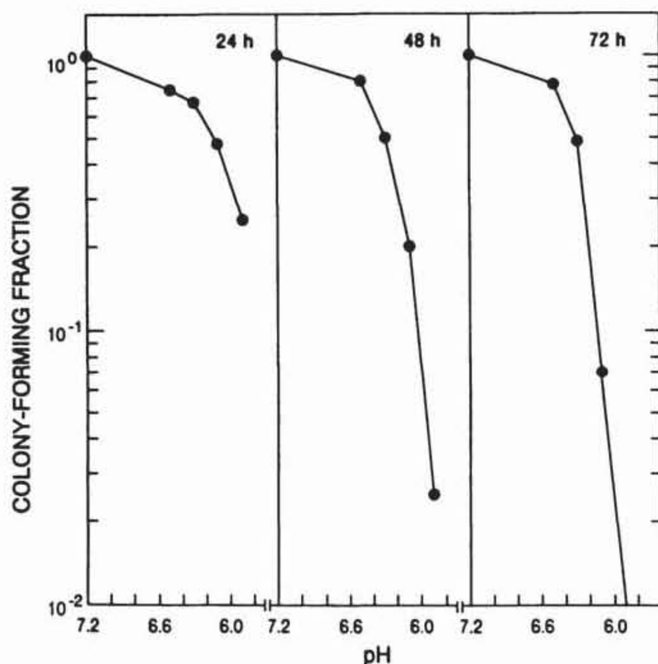

Fig. 1. Colony-forming fraction of M1R rat mammary carcinoma cells following incubation in drug-free DMEM at different pH_e for 24–72 h. At pH_e 5.9 the colony-forming fraction following a 72-h incubation was 0.004 (not shown).

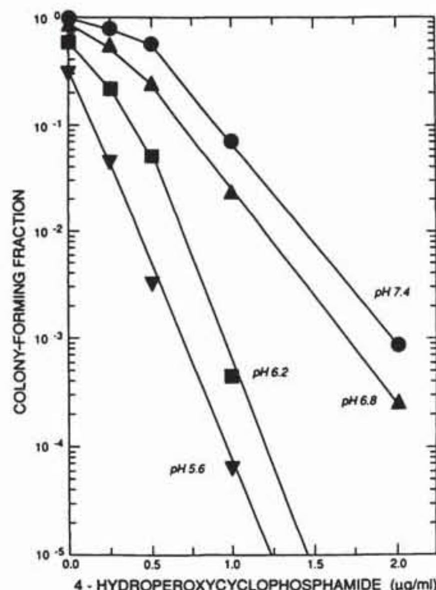

Fig. 2. Cytotoxic effect of 4-OOH-CP on M1R cells as a function of pH_e.

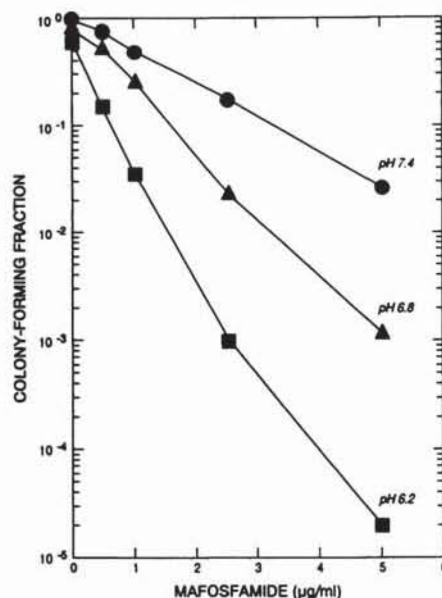

Fig. 3. Inhibition of colony-forming capacity of M1R cells exposed to MAFO at different pH_e.

The kinetics of inhibition of colony-forming capacity by aCP were investigated using MAFO as a source of 4-OH-CP. The fraction of colony-forming cells decreased as a function of the time of drug exposure both at pH_e 7.4 and at pH_e 6.2 (Fig. 4). After drug exposure for 12 h (24 h) about 65% (100%) of the respective maximum inhibitory effect was obtained at both pH_e values. Incubation of cells with MAFO for periods >24 h did not result in additional cytotoxicity at either pH_e in comparison to 24-h exposure.

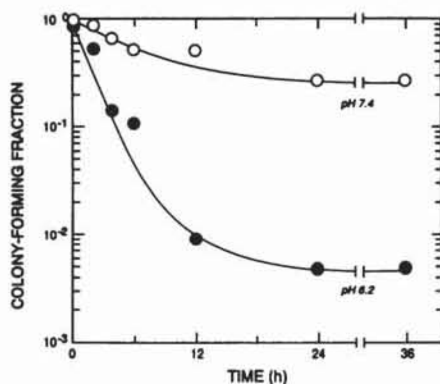

Fig. 4. Kinetics of inhibition of colony-formation of M1R cells exposed to MAFO (2 μg/ml) for up to 36 h at different pH_e.

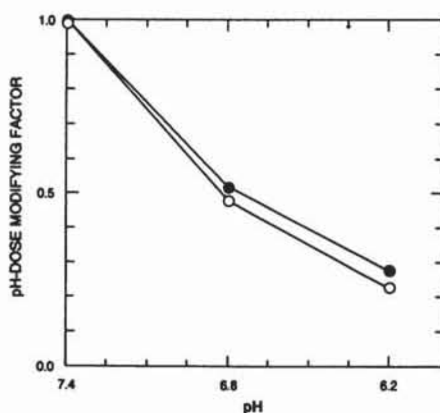

Fig. 5. Sensitivity of M1R cells to MAFO as a function of pH_e. The "pH-dose modifying factor" (ordinate) is defined as the MAFO concentration required to decrease the colony-forming fraction to a defined level at a given pH_e, divided by the MAFO concentration required for the same cytotoxic effect at pH_e 7.4. Data are presented for colony-forming fractions reduced to 10% and 1%, respectively. ●, colony-forming fraction (1%); ○, colony-forming fraction (10%).

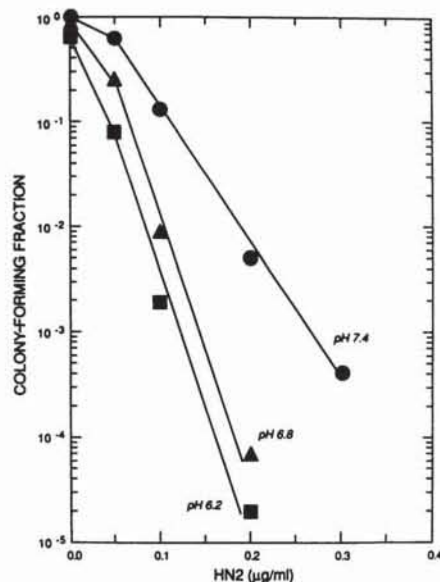

Fig. 6. Cytotoxic effect of HN2 on M1R cells at different pH_e.

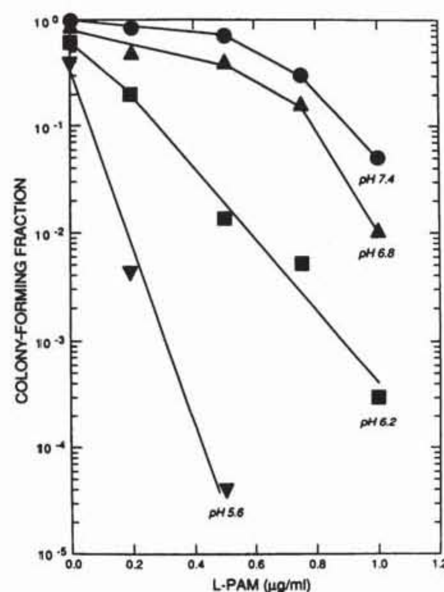

Fig. 7. Colony-forming fraction of M1R cells following exposure to L-PAM at pH_e 7.4 *versus* acidic culture media.

pH_e as a Dose Modifying Factor. From the data obtained with MAFO as the precursor of 4-OH-CP, a "pH-dose modifying factor" (pH-DMF) was calculated. The pH-DMF indicates the drug concentration required at acidic pH_e to achieve a given level of cytotoxicity (*e.g.*, colony-forming fraction 10% or 1% of untreated control) divided by the drug concentration resulting in an identical fraction of colony-forming cells at pH_e 7.4. As shown in Fig. 5, only 20% of the MAFO concentration required to reduce the fraction of colony-forming cells to 1% at pH_e 7.4 are sufficient to achieve the same effect at pH_e 6.2 (pH-DMF = 0.2).

Effect of Acidic Microenvironment on the Cytotoxic Action of Chloroethylating Drugs Structurally Different from CP. In addition to CP a number of chloroethylating agents have gained wide clinical application. Among these are HN2, L-PAM, and chlorambucil. As with CP, the action of these drugs resides in their bis-chloroethylamine group. In IFO, an oxazaphosphorine analog of CP, one of the chloroethyl side chains is shifted from the amino nitrogen to the ring nitrogen. The results of exposure of M1R cells to these agents in normal and acidic culture media are demonstrated in Figs. 6–9. The cytotoxic action of HN2, L-PAM, and chlorambucil was markedly enhanced when pH_e was lowered from 7.4 to 6.2. At a concentration of 0.2 μg/ml, the cytotoxic effect of HN2 at pH_e 6.2 was potentiated by about a factor of 200 as compared to pH_e 7.4. In contrast, the colony-forming capacity of cells treated with IFO remained essentially unaffected by variations of pH_e over a range of 7.4 to 5.6. The bis-chloroethylamine group may thus be a critical determinant for H^+ ion-mediated enhancement of cytotoxicity in this group of agents.

Molecular Mechanisms Sensitive to H^+ Ion-mediated Modulation of Alkylating Drug Cytotoxicity. Nor-HN2 is one of the terminal metabolites of aCP (14). In a cell free *in vitro* assay system, the alkylating potency of nor-HN2 has been shown to be highly sensitive to alterations of pH (14, 22). To evaluate the role of CP metabolites in H^+ ion-mediated enhancement of CP cytotoxicity, the effect of pH_e on the cytotoxic action of nor-HN2 was investigated. At pH_e 6.2 the cytotoxic effect of nor-NH2 on M1R cells (10 μg/ml) was potentiated by a factor of about 5×10^3 as compared to pH_e 7.4 (Fig. 10).

In all forementioned experiments drug cytotoxicity was analyzed as a function of pH_e. However, the critical molecular targets of alkylating drugs are located intracellularly, in particular in the nucleus (DNA). We therefore investigated whether alterations of pH_e are accompanied by corresponding shifts of pH_i. For these experiments the culture medium of M1R monolayers, previously grown at pH_e 7.4, was changed to medium of

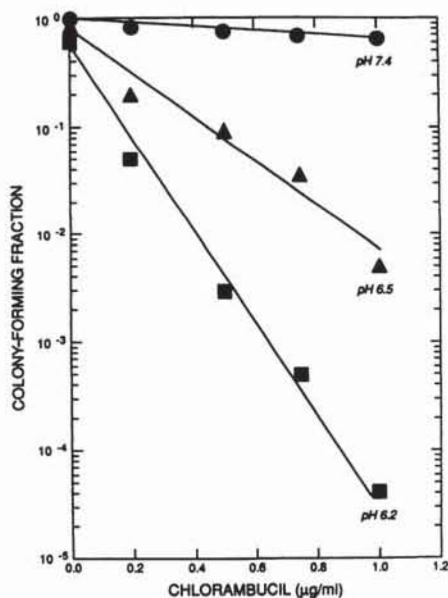

Fig. 8. Cytotoxicity of chlorambucil in M1R cells at different pH_e.

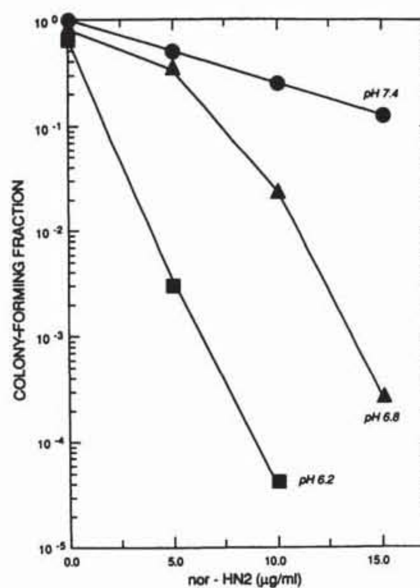

Fig. 10. H^+ ion-mediated enhancement of nor-HN2 cytotoxicity in M1R cells.

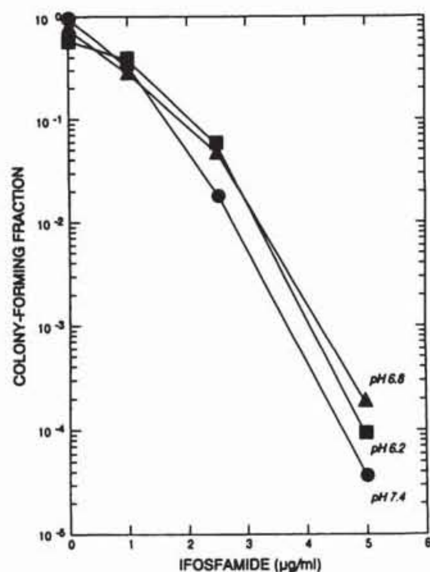

Fig. 9. Colony-forming fraction of M1R cells exposed to ifosfamide at different pH_e.

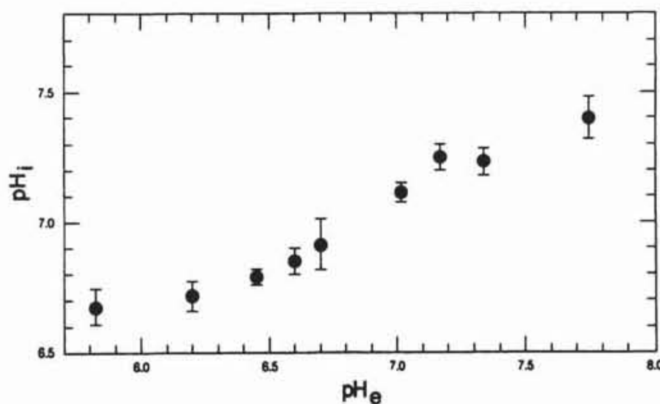

Fig. 11. Relationship between pH_e and pH_i in M1R cells. Each point represents the mean of 3–13 measurements (±SD).

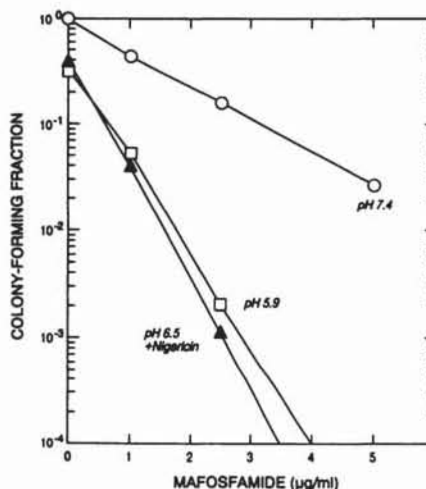

Fig. 12. Dependence of MAFO cytotoxicity on pH_i. The colony-forming fraction of M1R cells exposed to MAFO with or without simultaneous exposure to the ionsphor nigericin was measured as a function of pH_e.

pH_e 5.8–7.8. Two h later pH_i in single cells was measured with the use of H^+ ion-sensitive microelectrodes (see "Materials and Methods"). In separate experiments (not shown) it had been demonstrated that a 2-h incubation at pH_e different from 7.4 was sufficient for generation of a new steady state relationship between pH_e and pH_i. As demonstrated in Fig. 11, the pH_i of M1R cells varied with pH_e; however, pH_i shifts were generally less pronounced than the variations of pH_e. For example, a pH_e of 7.8 corresponded to pH_i of 7.4, and a pH_e 6.2 resulted in a shift of pH_i to 6.7.

In a further set of experiments two different techniques were applied, each of which lowered the pH_i of MAFO-treated cells to approximately the same value (pH_i 6.5–6.6) while the corresponding pH_e values were different (5.9 *versus* 6.5, respectively). If pH_i was the primary determinant for MAFO-induced cytotoxicity, then both modalities would be expected to give similar results. First, pH_i was lowered solely by reduction of pH_e. M1R cells were exposed to MAFO at pH_e 5.9, corresponding to a pH_i of ~6.6 (Fig. 11). Alternatively, pH_i was equilibrated with pH_e (6.5) prior to drug exposure, using the H^+ ion ionophor nigericin (23). Fig. 12 demonstrates that after exposure to MAFO at pH_e 5.9 (pH_i ~6.6) the fraction of colony-forming M1R cells did not differ significantly from the fraction measured after exposure to MAFO at pH_e 6.5 in the presence

of nigericin (pH_i 6.5). These results support the view that the cytotoxic effect of aCP is increased primarily by raising the intracellular concentration of H^+ ions (which is sensitive to variations of pH_e). At the concentration used (0.25 μg/ml) nigericin *per se* was only slightly cytotoxic: the fraction of colony-forming cells at pH_e 6.5 was reduced by 60% as compared to a reduction by 20% at pH_e 6.5 without nigericin.

The cytotoxic action of bifunctional chloroethylating agents is considered to be mainly dependent on the formation of DNA monoadducts (O^6-alkylguanine), with the varying cellular capacity for their removal and repair as a modulating determinant, and the subsequent formation of cytotoxic DNA interstrand cross-links (diadducts; 24, 25). DNA diadducts are thus formed in a two-step process. Following covalent binding of one of the chloroethyl groups to a single nucleophilic site in one DNA strand the second chloroethyl group is attached to the complementary strand after a time interval which appears to be drug specific (26–28). DNA interstrand cross-links may subsequently be removed by enzymatic repair mechanisms (26–28). To investigate the relative sensitivities of these reactions to alterations of pH, M1R cells were treated with L-PAM. The formation and removal of DNA cross-links following exposure to this drug have been studied in detail (26). During the 1-h period of exposure to L-PAM mainly DNA monoadduct formation takes place; at the end of drug exposure the "cross-linking index" is low. This index increases after removal of the drug, reaching a maximum 12 h later. The amount of cross-links detectable in the DNA of surviving cells then begins to decrease, reaching pretreatment values at about 40 h after the end of drug exposure. Similar observations have been reported for aCP, 4-OOH-CP, and for 4-sulfido-cyclophosphamides (27–29). During the phase predominated by DNA monoadduct formation, as analyzed after a 1-h exposure of M1R cells to L-PAM, an acidic microenvironment (pH_e 5.6) resulted in a marked enhancement of cytotoxicity as compared to exposure at pH_e 7.4 (Fig. 13). The effect of reduced pH_e during the period mainly characterized by DNA cross-link formation was investigated by incubating M1R cells previously exposed to L-PAM for 1 h at pH_e 7.4 for a further 14 h at either pH_e 7.4 or pH_e 5.6 in drug-free medium before determining the fractions of colony-forming cells. To insure complete removal of free L-PAM, the cells were washed three times with complete culture medium. The resulting increase in drug cytotoxicity at reduced pH_e was less pronounced than in the foregoing experiments. At pH_e 5.6 the fraction of colony-forming M1R cells was 0.007 as compared to 0.04 at pH_e 7.4 (L-PAM concentration, 6 μg/ml). For analysis of the effect of reduced pH_e on aCP-treated cells during the subsequent phase (DNA cross-link removal and repair), M1R cells were again exposed to L-PAM for 1 h (pH_e 7.4), washed, and incubated in drug-free medium (pH_e 7.4) for 14 h. Thereafter, pH_e in part of the culture plates was lowered to 5.6 for 28 h. Then the cells were plated to determine the respective fractions of colony-forming cells. As shown in Fig. 13, a reduction of pH_e during this phase did not result in additional toxicity in comparison to the counterpart cells kept at pH_e 7.4 during the entire posttreatment period.

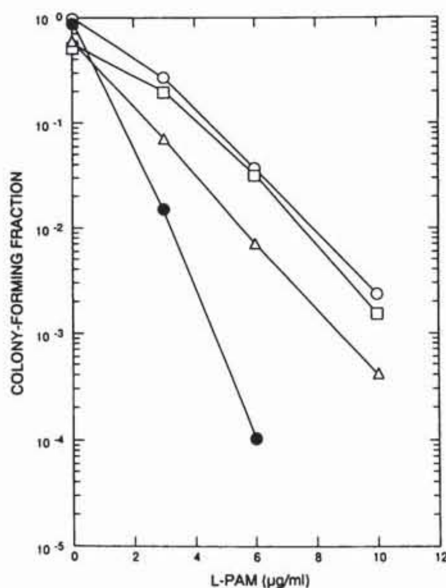

Fig. 13. Cytotoxic effect of simultaneous or sequential exposure of M1R cells to L-PAM and reduced pH_e. ○, L-PAM (1 h, pH_e 7.4); ●, L-PAM (1 h, pH_e 5.6); △, L-PAM (1 h, pH_e 7.4, followed by 14-h incubation in drug-free medium at pH_e 5.6); □, L-PAM (1 h, pH_e 7.4, followed by drug-free incubation at pH_e 7.4 for 14 h, and subsequently for 28 h at pH_e 5.6).

DISCUSSION

Any strategy to be considered for improving the effectiveness of cancer chemotherapy must rely on the exploitation of phenotypic differences between normal and malignant cells. One such difference is the ability of most malignant cells, as opposed to unperturbed normal cells, to aerobically convert glucose to lactic acid at a rate dependent on the microenvironmental glucose concentration (3, 4, 6). In a previous study we have shown that parenteral administration of glucose to tumor-bearing rats increases the rate of aerobic glycolysis in malignant cells, which in turn leads to an elevated concentration of lactic acid selectively in the tumor tissue (10). This observation is consistent with results reported by other investigators. In tumors of different histological type and of both animal and human origin, the concentration of H^+ ions can thus be increased by a factor of up to 10 (Table 1), while pH distributions remain essentially unchanged in normal tissues of hyperglycemic (nondiabetic) tumor-bearing hosts (Table 2).

Nitrogen mustard derivatives like CP and its analogues represent one of the most effective classes of anticancer agents. The cytotoxic effects of bis-chloroethylating agents are concentration-dependent both *in vitro* and *in vivo* (30). Theoretically, therefore, the therapeutic efficiency of these agents could be improved by either increasing their concentration or their reactivity (on a molar basis) selectively in malignant tissues. The work presented here is an example of the latter approach: extracellular, and the subsequent intracellular reduction of pH strongly increases the cytotoxicity of various bis-chloroethylating agents. The average pH_e in normal tissues is in the range of 7.0 to 7.4 (Table 2). After exposure to aCP at pH_e 7.4 in culture, the fraction of colony-forming M1R cells decreased to 8% of untreated control cells. However, when pH_e was lowered to 6.2 (approximately the mean "aggregated" pH in malignant tumors of hyperglycemic hosts; 10; Table 1), the fraction of colony-forming cells decreased to 0.04%. This corresponds to a more than 100-fold potentiation of aCP cytotoxicity at pH_e 6.2. The cytotoxic effect of aCP was even more enhanced when pH_e was lowered to the minimum level measured in different areas of transplanted tumors in glucose-perfused rats (5.3–6.1; 10). At pH_e 5.6 the cytotoxic effect of aCP was potentiated by a factor of 10^3. Similar results were obtained with HN2, nor-HN2, L-PAM, and chlorambucil.

DNA is considered a critical molecular target of bifunctional alkylating drugs (24, 25). Thus pH_i, in particular intranuclear pH_i, may play a critical role in the H^+ ion-mediated potentiation of drug cytotoxicity. This notion is supported by the results of

the present experiments, including those using the ionophor nigericin to equilibrate pH_i with pH_e. The fraction of colony-forming M1R cells decreased to the same level irrespective of whether pH_i was lowered to 6.5–6.6 by reduction of pH_e to 6.5 in the presence of nigericin, or by decreasing pH_e to 5.9 in the absence of the ionophor. Within the pH_e range studied (5.8–7.8), the corresponding shifts of pH_i were somewhat smaller than the changes of pH_e, indicating the cells' ability to keep pH_i closer to the physiological value in the presence of a lower pH_e, in agreement with data published by other investigators (23, 31, 50). pH_i values may be nearer pH_e in the case of malignant cells actively producing lactic acid in the presence of high concentrations of glucose (as opposed to the present experiments where the lowering of pH_i was achieved by reducing pH_e). In our analyses, pH_e 6.2, a pH value frequently used in this study, lead to a pH_i of 6.7 in M1R cells. This value is higher than the pH_i reported for actively glycolyzing cells both *in vitro* and *in vivo* (32, 33). When the glucose supply *in vitro* to malignant cells previously starved of glucose was sharply raised, a procedure resembling the elevation of glucose concentration in tumor interstitial fluid after parenteral administration of glucose (8), pH_i decreased from 6.9 to 6.3 (32). Conversely, when glucose was injected i.p. into tumor-bearing mice, the pH_i of Rif-1 tumor cells, as measured by nuclear magnetic resonance spectroscopy, decreased to 6.6 (33). The potentiation of aCP cytotoxicity at reduced pH_e (6.2) reported here, thus underestimates the enhancement expected at an "aggregated" acidic pH of 6.2 in malignant tumors *in vivo* (for detailed reviews of pH distributions in different compartments of mammalian tissues, see Refs. 10 and 34, and literature cited therein).

The H^+ ion-mediated enhancement of aCP cytotoxicity is not due to a more rapid formation of the reactive alkylating metabolites from 4-OH-CP at reduced pH_e as compared to physiological culture medium. Wagner *et al.* (35) have shown that in aqueous solutions the decomposition of 4-OH-CP to phosphoramide mustard and acrolein is in fact prolonged at pH 5.5 as compared to pH 7.3. This does, however, not imply that the increased cytotoxicity of aCP at low pH_e can be explained by extended exposure of cells to the more slowly generated reactive metabolites, since at both pH_e 7.4 and pH_e 6.2 maximum inhibition of colony-formation was observed after 24-h exposure to the drug.

Phosphoramide mustard is considered to be the major reactive metabolite of aCP (14, 22). Using the nitrobenzylpyridine assay for measuring the alkylating activity of CP metabolites, it has been shown that the reactivity of phosphoramide mustard was only slightly increased when the pH of the assay solution was lowered below physiological values (14, 22). In contrast, the reactivity of nor-HN2 was highly sensitive to variations of pH (14, 22). At pH 7.4 alkylation of nitrobenzylpyridine by nor-NH2 was marginal or not detectable. Under acidic conditions, however, the alkylating activity of nor-HN2 was strongly enhanced and exceeded that of phosphoramide mustard. We propose, therefore, that the H^+ ion-mediated potentiation of aCP cytotoxicity mainly resides with this metabolite. This is supported by our finding that the cytotoxic action of 4-OOH-IFO is not H^+ ion-dependent. Primary metabolites of IFO with structural similarities to those of CP have been identified (36). However, ifosfamide mustard does not, as phosphoramide mustard, further decompose to yield metabolites analogous to nor-HN2, since one of the chloroethyl side chains of IFO is linked to the phosphate group and not to the amino nitrogen as in CP and its metabolites.

In the present experiments, the cytotoxicity of HN2 was also pH-dependent. In the nitrobenzylpyridine assay, on the other hand, HN2 reactivity was not significantly enhanced when the pH value was shifted from 7.4 to 5.6 (14). This discrepancy can be explained by the known enzymatic demethylation of HN2 to the H^+ ion-sensitive nor-HN2 in living cells (37).

From the results of simultaneous or sequential exposure of M1R cells to L-PAM and to an acidic microenvironment, we conclude that the phase of drug uptake and the formation of DNA monoadducts is most pH-sensitive. To a lesser extent, DNA interstrand cross-link formation also appears to be enhanced at acidic pH. The overall results are in agreement with data reported for EMT6 tumor cells exposed to mitomycin C (38), where an increased rate of DNA cross-link formation was observed when pH_e was lowered from 7.5 to 5.7, 1 h prior to and during a 2-h drug exposure. In the present study, a reduction of pH_e during the phase of DNA cross-link removal and repair did not significantly influence the cytotoxic effect of L-PAM. The molecular processes occurring during this phase may thus be the least H^+ ion-sensitive mechanisms in the sequence of events following the interaction of bis-chloroethylating agents with target cells.

Hahn and Shiu have analyzed the response of Chinese hamster cells to antineoplastic drugs different from oxazaphosphorines as function of pH_e either alone or in combination with hyperthermia (51, 52). Whereas the cytotoxic effect of methotrexate, BCNU and bleomycin was not H^+ ion-dependent in the pH range of 6.5 to 8.5 at 37°C, both BCNU and bleomycin cytotoxicity exhibited a marked pH-sensitivity in combination with simultaneous heat exposure (43°C).

When M1R cells were exposed to aCP at pH_e 6.2, the "pH-dose modifying factor" was 0.2 relative to exposure to aCP at pH_e 7.4. Solely by lowering pH_i, a cytotoxic effect can thus be exerted which, under physiological conditions, would require a five times higher dose. The magnitude of this dose-sparing effect makes a tumor-selective downshift of pH by glucose perfusion a candidate procedure as an adjunct to systemic chemotherapy with alkylating agents; in particular, since the "drug" (glucose) used for sensitization is nontoxic. The feasibility of this approach will, however, depend on whether the pH in primary human tumors can be shifted to the same level and for the same duration as the pH in transplanted rodent tumors. We are aware of only two reports on pH measurements in human tumors following glucose administration (11, 45). In these studies hyperglycemia was generated only by either oral or short term (~1 h) i.v. glucose administration. Mean blood glucose did not exceed 23 and 16 mmol/liter, respectively, and the mean intratumoral pH was only lowered to 6.6–6.7. Studies on animal tumors indicate, however, that tumor pH may be inversely correlated with serum glucose up to 50 mmol/liter (10). In addition, there is evidence that the duration of glucose administration may be an important determinant for the pH response of neoplastic tissues (10, 41). It remains to be investigated, therefore, whether intratumoral pH values of 6.2–6.5 can be generated also in humans by intensification of i.v. glucose administration. In particular, it has to be analyzed whether in human tumors an acidosis can be maintained long enough to allow for H^+ ion-mediated drug activation (~12 h). It is of interest, therefore, that in transplanted rat tumors mean pH values of 6.1–6.2 could be maintained up to 48 h by continuous glucose administration (10). Conversely, in healthy volunteers serum glucose concentrations of ~30 mmol/liter have been generated for up to 24 h solely by i.v. glucose infusion (39, 40). Apart from osmotic diuresis no serious side effects were observed.

Some conflicting results have been reported regarding the effect of combinations of alkylating drugs with parenteral ad-

ministration of glucose *in vivo*. Whereas Osinsky *et al.* (41) reported partial and complete remissions of Guerin carcinomas following treatment with thiophosphamide and i.v. glucose administration, Urano and Kim (42) noted no significant enhancement of cytotoxicity against a murine fibrosarcoma when CP treatment was combined with i.p. glucose administration; however, no information on intratumoral pH was given. The discrepancy between the latter data and the results presented here may be explained by the short duration of intratumoral acidosis following single i.p. injections of glucose into tumor-bearing mice (43), a technique applied in the study by Urano and Kim (42). Investigations on the treatment of transplanted tumors with chloroethylating drugs combined with tumor-selective acidification are in progress in our laboratory.

ACKNOWLEDGMENTS

We are indebted to Professor H. D. Waller for generous support throughout this investigation and to S. Conzelmann and W. Drosdziok for excellent technical assistance.

REFERENCES

1. Ross, W. C. J. Increased sensitivity of the Walker tumour towards aromatic nitrogen mustards carrying basic side chains following glucose pretreatment. Biochem. Pharmacol., *8:* 235–240, 1961.
2. Yatvin, M. B., Kreutz, W., Horwitz, B. A., and Shinitzky, M. pH-Sensitive liposomes: Possible clinical implications. Science (Wash. DC), *210:* 1253–1254, 1980.
3. Warburg, O. The metabolism of tumors. London: Arnold Constable, 1930.
4. Aisenberg, A. C. The glycolysis and respiration of tumors. New York and London: Academic Press, 1961.
5. Pouysségur, J., Franchi, A., Salomon, J-C., and Silvestre, P. Isolation of a Chinese hamster fibroblast mutant defective in hexose transport and aerobic glycolysis: its use to dissect the malignant phenotype. Proc. Natl. Acad. Sci. USA, *77:* 2698–2701, 1980.
6. Eagle, H., Barban, S., Levy, M., and Schulze, H. O. The utilization of carbohydrates by human cell cultures. J. Biol. Chem., *233:* 551–558, 1958.
7. Gullino, P. M., Grantham, F. H., and Courtney, A. H. Glucose consumption by transplanted tumors *in vivo*. Cancer Res., *27:* 1031–1040, 1967.
8. Gullino, P. M., Clark, S. H., and Grantham, F. H. The interstitial fluid of solid tumors. Cancer Res., *24:* 780–794, 1964.
9. Rauen, H. M., Friedrich, M., and Norpoth, K. Messungen zur Manipulation der Glucose-abhängigen Tumorsäuerung *in vivo*. Z. Naturforsch., *23b:* 1461–1475, 1968.
10. Jähde, E., and Rajewsky, M. F. Tumor-selective modification of cellular microenvironment *in vivo*: effect of glucose infusion on the pH in normal and malignant rat tissues. Cancer Res., *42:* 1505–1512, 1982.
11. Ashby, B. S. pH-Studies in human malignant tumours. Lancet, *2:* 312–315, 1966.
12. Wheeler, G. P. Alkylating agents. *In:* J. F. Holland and E. Frei III. (eds.), Cancer Medicine, pp. 791–805. Philadelphia: Lea and Febiger, 1974.
13. Warwick, G. P. The mechanism of action of alkylating agents. Cancer Res., *23:* 1315–1333, 1963.
14. Colvin, M., Brundrett, R. B., Kan, M-N. N., Jardine, I., and Fenselau, C. Alkylating properties of phosphoramide mustard. Cancer Res., *36:* 1121–1126, 1976.
15. Begg, A. C., Shrieve, D. C., Smith, K. A., and Terry, N. H. A. Effects of hypoxia, pH, and growth stage on cell killing in Chinese hamster V79 cells *in vitro* by activated cyclophosphamide. Cancer Res., *45:* 3454–3459, 1985.
16. Rajewsky, M. F., and Grüneisen, A. Cell proliferation in transplanted rat tumors: influence of the host immune system. Eur. J. Immunol., *2:* 445–447, 1972.
17. Matsushima, Y., Kanzawa, F., Hoshi, A., Shimizu, E., Nomori, H., Sasaki, Y., and Sajio, N. Time-schedule dependency of the inhibiting activity of various anticancer drugs in the clonogenic assay. Cancer Chemother. Pharmacol., *14:* 104–107, 1985.
18. Ammann, D., Lanter, F., Steiner, R. A., Schulthess, P., Shijo, Y., and Simon, W. Neutral carrier based hydrogen ion selective microelectrode for extra- and intracellular studies. Anal. Chem., *53:* 2267–2269, 1981.
19. Duffey, D. E. Intracellular pH and bicarbonate activities in rabbit colon. Am. J. Physiol., *246*(Cell Physiol. 15): C558–C561, 1984.
20. Redwood, W. R., Hilton, J., Colvin, M., and Owens, A. H. The cellular uptake of 4-hydroperoxycyclophosphamide. Proc. Am. Assoc. Cancer Res., *23:* 169, 1982.
21. Niemeyer, U., Enger, J., Scheffler, G., Molge, K., Sauerbier, D., and Weigert, W. Chemical characterization of ASTA Z 7557 (INN mafosfamide, *cis*-4-sulfoethylthiocyclophosphamide) a stable derivative of 4-hydroxycyclophosphamide. Invest. New Drugs, *2:* 133–139, 1984.
22. Struck, R. F., Kirk, M. C., Witt, M. H., and Laster, W. R., Jr. Isolation and mass spectral identification of blood metabolites of cyclophosphamide: evidence for phosphoramide mustard as the biologically active metabolite. Biomed. Mass Spectrometry, *2:* 46–52, 1975.
23. Rotin, D., Wan, P., Grinstein, S., and Tannock, I. Cytotoxicity of compounds that interfere with the regulation of intracellular pH: a potential new class of anticancer drugs. Cancer Res., *47:* 1497–1504, 1987.
24. Kohn, K. W. DNA as a target in cancer chemotherapy: measurement of macromolecular DNA damage produced in mammalian cells by anticancer agents and carcinogens. Methods Cancer Res., *16:* 291–345, 1979.
25. Colvin, M. The alkylating agents. *In:* B. Chabner (ed.), Pharmacologic Principles of Cancer Treatment, pp. 276–308. Philadelphia: W. B. Saunders Co., 1982.
26. Hansson, J., Lewensohn, R., Ringborg, U., and Nilsson, B. Formation and removal of DNA cross-links induced by melphalan and nitrogen mustard in relation to drug-induced cytotoxicity in human melanoma cells. Cancer Res., *47:* 2631–2637, 1987.
27. Erickson, L. C., Ramonas, L. M., Zaharko, D. S., and Kohn, K. W. Cytotoxicity and DNA cross-linking activity of 4-sulfidocyclophosphamides in mouse leukemia cells *in vitro*. Cancer Res., *40:* 4216–4220, 1980.
28. Hilton, J. Deoxyribonucleic acid crosslinking by 4-hydroperoxycyclophosphamide in cyclophosphamide-sensitive and -resistant L1210 cells. Biochem. Pharmacol., *33:* 1867–1872, 1984.
29. Crook, T. R., Souhami, R. L., and McLean, A. E. M. Cytotoxicity, DNA cross-linking, and single strand breaks induced by activated cyclophosphamide and acrolein in human leukemia cells. Cancer Res., *46:* 5029–5034, 1986.
30. Frei, E., III, and Canellos, G. P. Dose: a critical factor in cancer chemotherapy. Am. J. Med., *69:* 585–594, 1980.
31. Bowen, J. W., and Levinson, C. H^+-transport and the regulation of intracellular pH in Ehrlich ascites tumor cells. J. Membrane Biol., *79:* 7–18, 1984.
32. Desmoulin, F., Galons, J-P., Canioni, P., Marvaldi, J., and Cozzone, P. J. ^{31}P Nuclear magnetic resonance study of a human colon adenocarcinoma cultured cell line. Cancer Res., *46:* 3768–3774, 1986.
33. Evelhoch, J. L., Sapareto, S. A., Jick, D. E., and Ackerman, J. J. H. *In vivo* metabolic effects of hyperglycemia in murine radiation-induced fibrosarcoma: a ^{31}P NMR investigation. Proc. Natl. Acad. Sci. USA, *81:* 6496–6500, 1984.
34. Roos, A., and Boron, W. F. Intracellular pH. Physiol. Rev., *61:* 296–434, 1981.
35. Wagner, T., Heydrich, D., Jork, T., Voelcker, G., and Hohorst, H. J. Comparative study on human pharmacokinetics of activated ifosfamide and cyclophosphamide by a modified fluorometric test. J. Cancer Res. Clin. Oncol., *100:* 95–104, 1981.
36. Colvin, M. The comparative pharmacology of cyclophosphamide and ifosfamide. Semin. Oncol., *9:* 2–7, 1982.
37. Ross, W. C. J. Biological alkylating agents. London: Butterworth, 1962.
38. Kennedy, K. A., McGurl, J. D., Leonardis, L., and Alabaster, O. pH Dependence of mitomycin C-induced cross-linking activity in EMT6 tumor cells. Cancer Res., *45:* 3541–3547, 1985.
39. Lippmann, H., and Graichen, D. Glucose- und K^+-Bilanz während hochdosierter intravenöser Glucosezufuhr. Infusionstherapie, *4:* 166–178, 1977.
40. Foerster, H. Fruktose und Sorbit als energieliefernde Substrate für die parenterale Ernährung. Infusionstherapie, *14:* 98–109, 1987.
41. Osinsky, S., Bubnovskaja, L., and Sergienko, T. Tumour pH under induced hyperglycemia and efficacy of chemotherapy. Anticancer Res., *7:* 199–202, 1987.
42. Urano, M., and Kim, M. S. Effect of hyperglycemia on thermo-chemotherapy of a spontaneous murine fibrosarcoma. Cancer Res., *43:* 3041–3044, 1983.
43. Tobari, C., Van Kersen, I., and Hahn, G. M. Modification of pH of normal and malignant mouse tissue by hydralazine and glucose, with and without breathing of 5% CO_2 and 95% air. Cancer Res., *48:* 1543–1547, 1988.
44. Von Ardenne, M., and Reitnauer, P. G. Über manipulierte Übersäuerung autochthoner Tumoren. Onkologie, *1:* 85–88, 1978.
45. Thistlethwaite, A. J., Alexander, G. A., Moylan, D. J., and Leeper, D. B. Modification of human tumor pH by elevation of blood glucose. Int. J. Radiat. Oncol. Biol. Phys., *13:* 603–610, 1987.
46. Reitnauer, P. G. Zur Methode der Übersäuerung von Tumoren *in vivo*. Z. med. Labortechn., *13:* 5–37, 1972.
47. Dickson, J. A., and Calderwood, S. K. Effects of hyperglycemia and hyperthermia on the pH, glycolysis, and respiration of the Yoshida sarcoma *in vivo*. J. Natl. Cancer Inst., *63:* 1371–1381, 1979.
48. Wike-Hooley, J. L., van den Berg, A. P., van der Zee, J., and Reinhold, H. S. Human tumour pH and its variations. Eur. J. Cancer Clin. Oncol., *21:* 785–791, 1985.
49. Jain, R. K., Shak, S. A., and Finney, P. Continuous noninvasive monitoring of pH and temperature in rat Walker 256 carcinoma during normoglycemia and hyperglycemia. J. Natl. Cancer Inst., *73:* 429–436, 1984.
50. Gonzalez-Mendez, R., Wemmer, D., Hahn, G., Wade-Jardetzky, N., and Jardetzky, O. Continuous-flow NMR culture system for mammalian cells. Biochim. Biophys. Acta, *720:* 274–280, 1982.
51. Hahn, G. M., and Shiu, E. C. Effect of pH and elevated temperatures on the cytotoxicity of some chemotherapeutic agents on Chinese hamster cells *in vitro*. Cancer Res., *43:* 5789–5791, 1983.
52. Hahn, G. M., and Shiu, E. C. Adaptation to low pH modifies thermal and thermo-chemical responses of mammalian cells. Int. J. Hyperthermia, *2:* 379–387, 1986.