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Perspectives in Cancer Research

Acid pH in Tumors and Its Potential for Therapeutic Exploitation[1]

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The development of effective therapy for malignant disease has been hindered by the lack of consistent differences between tumor and normal tissue. Thus, unlike antibiotic treatment of bacteria, it has been difficult to develop therapeutic strategies which have major toxic effects against tumors, without causing damage to normal cells. One major difference between many solid tumors and surrounding normal tissue is the nutritional and metabolic environment. The functional vasculature of tumors is often inadequate to supply the nutritional needs of the expanding population of tumor cells, leading to deficiency of oxygen and many other nutrients. The production of lactic acid under anaerobic conditions and the hydrolysis of ATP in an energy-deficient environment contribute to the acidic microenvironment which has been found in many types of tumor.

Deficiency of nutrients and acid conditions may contribute to cell death and necrosis within solid tumors. Many cells, however, are known to survive under marginal conditions, and these cells may be an important cause for failure of conventional therapies. Thus cells remote from blood vessels may be resistant to radiation because of hypoxia and they may also be resistant to anticancer drugs because of limited drug access or because they have a low proliferative rate. Hypoxia and acidity represent two factors that might be exploited therapeutically to destroy such cells.

In the present article we review the evidence for development of acid pH within tumors. Regulation of pH_i[4] in the face of an acid load is considered in some detail, and the role of ion exchangers located in the cell membrane in maintaining cell viability and tumor growth is reviewed. Finally we describe the influence of pH on cell survival after conventional therapy and introduce the possibility that new therapies might be targeted against the membrane-based mechanisms that regulate pH_i under the acidic conditions that prevail in many tumors.

Tumor pH

Measurement of pH in Tissue. Most estimates of pH in tissue have been obtained by insertion of pH electrodes (1). Probes for measuring pH in tissue have been constructed with a tip diameter ranging from about 1 μm to a few mm. Measurements made by such electrodes are presumed to reflect predominantly the pH of the extracellular fluid, with an unknown component from damaged cells and blood released from ruptured capillaries (2). Most electrodes are large compared with the diameter of individual cells and cannot easily be used to study variation in pH over small distances within tumors. Very fine electrodes have been constructed (3) but are associated with a decrease in signal:noise ratio. Such electrodes have been used to determine pH as a function of depth in multicellular spheroids (4).

Recently, ^{31}P-NMR spectroscopy has been adapted to measure tissue pH. The method is based on the pH-dependent chemical shift of the resonance frequency due to phosphates. The relative concentrations of $H_2PO_4^-$ and HPO_4^{2-} are dependent on pH; due to rapid chemical exchange between these species ($H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$) the observed NMR spectrum consists of a single peak the chemical shift of which is pH dependent. Since phosphates are largely intracellular, the method leads to an estimate of pH_i. Adaptation of NMR to measure pH_i in tissue requires that an appropriate surface coil be placed over or around the tissue of interest (5–9). The method is sensitive and provides an average value for pH_i within the magnetic field, but at present it gives little information about spatial heterogeneity.

Estimates of pH within Tumors. A comprehensive review of several thousand microelectrode measurements of pH in human and animal tumors has been provided by Wike-Hooley *et al.* (10). Their results may be summarized as follows: (*a*) determination of pH in s.c. tissue and muscle ranged from 7.00 to 8.06, with mean values of 7.52 for human s.c. tissue and 7.32 and 7.43 for muscle in dogs and rats, respectively; (*b*) there is a wider range of pH values in malignant tissue, from about pH 5.8 to pH 7.6 in both human and rodent tumors. There is considerable variation within different regions of the same tumor; (*c*) in general, tumors are more acidic than normal tissues with median pH values of about 7.0 in tumors and 7.5 in normal tissues.

Cumulative distributions of pH obtained in tumors and normal tissues by Wike-Hooley *et al.* (10) are reproduced in Fig. 1.

In general, more recent estimates of the average pH_i in tumors obtained by ^{31}P-NMR are in the same range as estimates of pH_e obtained by insertion of electrodes. Exceptions include measurements of pH_i in some human brain tumors which may be more alkaline than the surrounding normal brain (9).

Several factors may influence the pH within tumors and normal tissues. In a further study of 105 human tumors, Wike-Hooley *et al.* (11) found greater acidity in primary tumors as compared to their metastases but no correlation between tumor pH and histology, degree of differentiation, tumor size, patient age, treatment history, or the presence or absence of ulceration. In contrast, Thistlethwaite *et al.* (12) found greater acidity in larger human tumors, but central regions were no more acidic than the periphery. In several studies of rodent tumors, the mean pH has been found to decrease with increasing tumor size. This effect has been observed with both electrode measurements of pH_e (13) and NMR measurements of pH_i (5–7), suggesting that it is not due to acidity in noncellular regions. Large regions of necrosis may in fact be relatively alkaline, due to breakdown of tissue and release of basic components of protein (14, 15).

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[4] The abbreviations used are: pH_i, intracellular pH; pH_e, extracellular pH; NMR, nuclear magnetic resonance; DMO, 5,5-dimethyl-2,4-oxazolidinedione; BCECF, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein; $[K^+]_i$, $[K^+]_e$, intracellular and extracellular concentration of potassium; $[Na^+]_i$, $[Na^+]_e$, intracellular and extracellular concentration of sodium; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; CCCP, carbonylcyanide-3-chlorophenylhydrazine; CHO, Chinese hamster ovary.

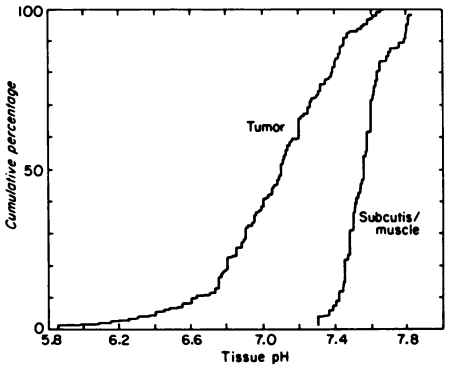

Fig. 1. Cumulative distribution of measurements of extracellular pH, obtained by electrode measurements, in tumors and normal tissues. Data reviewed by Wike-Hooley *et al.* (10); reproduced with permission.

The above observations are consistent with the tendency for the functional vasculature of tumors to decrease during growth (16, 17) leading to increased hypoxia, anaerobic metabolism, and acidosis. A correlation between hypoxia and low pH has been observed in some rodent tumors (3), and both pO_2 and pH have been observed to fall at increasing depths in spheroids and in some rodent tumors (14, 15).

Causes of Acidity within Tumors. Warburg (18) measured the production of lactic acid in slices of tumors and normal tissues and reported a consistently greater production of lactate in tumor tissue. On the basis of this observation, he proposed that tumor cells had impairment of respiration, since they depended on glycolysis for a source of metabolic energy. This hypothesis has not withstood the test of time but stimulated a large number of studies on tumor metabolism. In general, these studies have confirmed an increased rate of glycolysis in tumor cells as compared to normal cells (19) but have shown that malignant cells were fully able to use respiration as a source of metabolic energy (20, 21). Pouyssegur *et al.* (22, 23) showed that mutant cells defective in the glycolytic pathway were completely dependent on oxygen for survival but retained a similar capacity to grow tumors as wild-type cells. Although some cell types may utilize glycolysis to produce the majority of their ATP under aerobic conditions, it is probable that the increased rate of glycolysis and production of lactic acid that is observed in many tumors results from the existence of hypoxic regions in which cells are dependent on anaerobic glycolysis to obtain energy. Measurements made with oxygen probes, and a large number of radiobiological studies, have provided ample evidence for the existence of hypoxic regions in most solid tumors.

Anaerobic glycolysis leads to the formation of 2 mol of lactic acid and 2 mol of ATP for each mol of glucose utilized. Because lactic acid has a pK_a of 3.9, it is dissociated into a lactate anion and a proton at physiological pH. Careful consideration of the stoichiometry of the glycolytic pathway reveals that the net production of protons in this pathway is small and that the main source of protons during anaerobic metabolism at physiological pH originates from the hydrolysis of ATP. At any pH in the range of 6.8–8.0 a total of 2 protons is produced during one cycle of anaerobic glycolysis plus ATP hydrolysis (24). Production of protons by this mechanism is probably a major cause of acidity in tumors, although additional pathways may also lead to acid production. Steady state aerobic energy metabolism has no net effect on pH, since under physiological conditions respiration consumes the same number of protons as are produced by hydrolysis of ATP (24, 25).

The rate of glycolysis is dependent on environmental conditions. The pathway is inhibited at acidic pH, probably due to the inhibition of phosphofructokinase, the rate-limiting enzyme in glycolysis (26, 27). Transport of lactate out of the cell is also inhibited at low extracellular pH (28, 29). Accumulation of intracellular lactate because of severe acidosis, or because of inadequate blood flow to remove lactate from tumor tissue, will tend to inhibit glycolysis through end product inhibition of the glycolytic pathway.

Modifiers of Tumor pH. It is possible to decrease the pH in experimental tumors by measures which are designed to produce systemic acidosis or to modify tumor metabolism or blood flow. Systemic acidosis may be achieved by increasing the level of carbon dioxide in inspired air or by providing bicarbonate in the drinking water. The latter method led to a fall in pH (~0.3 unit) in the extracellular fluid of Walker rat tumors (30), but no fall in pH was observed in a different type of rat tumor (31).

A consistent decrease in tumor pH can be achieved by infusion of glucose, with or without insulin to stimulate its cellular uptake (2, 31, 32). The magnitude of the effect depends on the concentration of glucose, the duration of infusion, and the tumor under investigation, but a decrease of 0.5 pH unit or greater has been achieved in several experimental tumors. A similar effect has been achieved in patients after ingestion of 100 g glucose (33). The effect is usually associated with increased production of lactic acid and was not observed after infusion of nonmetabolized sugars such as galactose (34). Glucose infusion also leads to decreased blood flow, probably because of increased viscosity (2, 32), and i.p. injection of glucose into rodents leads to decreased blood volume because of osmotic shift of fluid into the peritoneal space (35). These effects lead to increased hypoxia and decreased clearance of lactate. Thus stimulation of glycolysis and decreased blood flow following glucose administration probably contribute to increased acidosis within different solid tumors.

Tumor blood flow may be inhibited by several drugs. Arterial vasodilators such as sodium nitroprusside or hydralazine may selectively increase the perfusion of normal tissues, leading to a corresponding decrease in the perfusion of tumors and a fall in their pH (36, 37). Recent experiments with hydralazine have shown that the drug can increase tumor hypoxia and enhance the effectiveness of hypoxia-specific drugs (38), but in another model system hydralazine led to a greater fall in pH of kidney and liver than of tumors (39). Von Ardenne and Reitnauer (40, 41) have reported that several additional compounds (amygdalin, β-glucosidase, and NAD) can enhance the effect of glucose to lower tumor pH, but given the variability among tumors these experiments need to be repeated in other systems before they are regarded as of general application.

In summary, there is firm evidence that solid tumors tend to be more acidic than normal tissues and that acidity can be enhanced by glucose administration with or without additional measures. Under such conditions, viability of cells is likely to depend critically on homeostatic mechanisms that maintain pH_i within the physiological range. In subsequent sections we will explore the mechanisms used by cells to regulate pH_i in the face of an acid load and the possible implications for tumor therapy.

Regulation of Intracellular pH

Measurement of pH_i

Studies of the regulation of pH_i require accurate methods for its measurement which respond rapidly to changes that occur in response to modification of the cellular environment. The

most direct method for measuring pH_i is through the use of microelectrodes, which can be inserted into selected large cells (*e.g.*, snail neurons, rat soleus muscle fibers) (42). These electrodes have a tip diameter of ~1 μm but are still too large to be inserted into most cells.

Until recently, the most widely used method for estimating pH_i in mammalian cells depended on the distribution of an isotopically labeled weak acid or base across the cell membrane. It is assumed that only the uncharged form is membrane permeant, and the equilibrium distribution on either side of the cell membrane can be calculated using the Henderson-Hasselbalch equation

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

where HA is the uncharged form and A^- is the proton acceptor (43, 44). The weak acid DMO has been used most often. In practice [^{14}C]DMO is used to determine its partition between the inside and the outside of the cell, with a parallel sample containing ^3H_2O and [^{14}C]polyethylene glycol used to determine cellular water content. The method can be applied widely but suffers from the following disadvantages (44): (*a*) cells are damaged during measurement, so that repeated measurements are not possible; (*b*) the slow distribution of DMO and other indicators renders the method inappropriate for studying rapid changes in pH_i; and (*c*) the method determines some average value of pH_i, and not cytoplasmic pH, inasmuch as weak acids are known to accumulate in organelles such as mitochondria.

The preferred method for measuring pH_i uses a pH-sensitive fluorescent dye such as the derivative of fluorescein BCECF (45). The uncharged acetoxy methyl ester of BCECF diffuses into the cell where nonspecific esterases cleave the ester groups, leaving the fluorescent, charged (and therefore impermeant) BCECF molecule. BCECF shows minimal leakage, and there is a linear relationship between its fluorescence intensity and pH_i within the range of 6.5–7.5. The conversion of fluorescence intensity to pH_i units is carried out with a calibration curve obtained by disrupting the cells with a detergent and recording the pH in medium following titration with concentrated acid or base solutions. It is necessary to correct for a slight red shift that occurs after the release of the dye by the detergent, and this can be estimated by use of the ionophore nigericin, which sets $pH_i = pH_e$ when cells are placed in K^+-containing solution such that $[K^+]_i = [K^+]_e$ (46, 47).

Rink *et al.* (45) have compared values of pH_i obtained using BCECF to those obtained by 3 other methods and have found them to be accurate and reproducible. The dye is excluded from organelles and therefore measures cytoplasmic pH. It allows a simple fluorimetric reading of pH_i with very rapid response time (see Fig. 2) and no apparent damage to cells. It can also be adapted for use with flow cytometry, allowing the sorting of cells with different pH_i (48) and for fluorescence imaging microscopy of intact tissue (49). By dissociating cells from tumors into HCO_3^--free medium containing amiloride (thereby blocking the major mechanisms for pH_i regulation, see below) followed by loading of cells with BCECF and flow cytometry, Hedley and Jorgensen (50) have attempted recently to measure the distribution of pH_i in experimental tumors.

Mechanisms Which Regulate pH_i

The main challenge faced by pH_i-regulatory mechanisms is relieving the cell of excess protons. This chronic acid loading originates from accumulation of metabolically generated acids

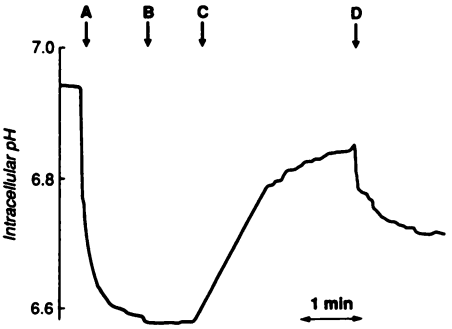

Fig. 2. Use of the fluorescent probe BCECF to study mechanisms which regulate pH_i. In the fluorimeter trace shown, cells were loaded with BCECF and then suspended in Na^+- and HCO_3^--free *N*-methyl-D-glucamine medium (58). At *Point A* cells were acidified with nigericin (2 μg/ml) (a process that can be achieved also by the ammonium prepulse method) and in *B* albumin (2.5 mg/ml) was added to bind excess nigericin. In *C* addition of NaCl (100 mM) allows exchange of extracellular Na^+ for intracellular H^+ leading to a rise in pH_i, which in *D* is blocked by adding amiloride (0.1 mM). The slope of the segment *CD* is a measure of Na^+/H^+ activity. Activity of the HCO_3^-/Cl^- ion exchange mechanisms can be measured in similar experiments where acid-loaded cells are added to bicarbonate-containing medium in the presence or absence of Na^+ and with amiloride present to inhibit Na^+/H^+ exchange.

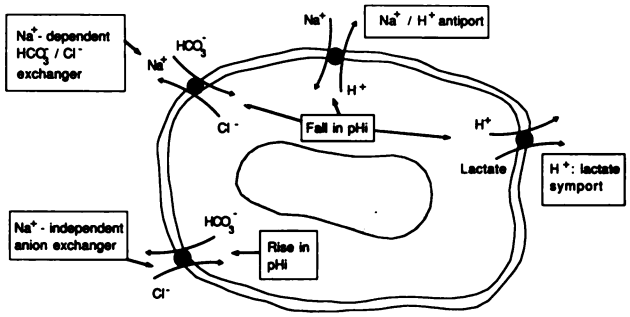

Fig. 3. Some of the mechanisms which are known to regulate intracellular pH in mammalian cells. When acid is produced within the cell, protons may be removed by the amiloride-sensitive Na^+/H^+ antiport or by an H^+:lactate symport, or they may be buffered by HCO_3^- which enters the cells via the Na^+-dependent HCO_3^-/Cl^- exchange agent. When pH_i tends to become alkaline HCO_3^- may be removed via the Na^+-independent anion exchanger. Additional mechanisms probably contribute to regulation of pH_i in some types of cell.

and from passive diffusion of H^+ (equivalents) into the cell due to the internally negative membrane potential. To overcome this chronic acidification, cells have evolved several methods to remove protons.

Short-term homeostasis of pH_i in cells involves the recruitment of rapid H^+-consuming mechanisms. These include physicochemical buffering, H^+-consuming metabolic reactions and transfer of acids from the cytosol into organelles [see Roos and Boron (44) for review]. Cells have also developed several membrane-based ion transport mechanisms for regulating pH_i, and these are illustrated schematically in Fig. 3. Major transport mechanisms which are known to contribute to regulation of pH_i in many types of cell include the Na^+/H^+ antiport, the Na^+-dependent HCO_3^-/Cl^- exchanger, and the cation-independent HCO_3^-/Cl^- exchanger (44, 51–53). The first two of these are involved primarily with regulation of pH_i in acid-loaded cells, whereas the latter probably participates in lowering pH_i of alkaline-loaded cells. Other membrane transporters may contribute to the regulation of pH_i by extruding protons from cells; examples include H^+ (ATPase) pumps often found in specialized epithelia and the lactate:proton symport (54).

In order to study the regulation of pH_i it is often necessary to induce intracellular acidification (or alkalinization) by artificial means and then observe the mechanisms which attempt to restore pH_i to the physiological range. One method is to use

ionophores such as nigericin which cause intracellular acidification by allowing entry of protons in exchange for K^+ which leaves the cells down its chemical gradient (46). An alternative method is the ammonium prepulse technique (55). Exposure of cells to NH_4^+ ($NH_4^+ \rightleftharpoons NH_3 + H^+$) causes initial cytoplasmic alkalinization due to entry of NH_3 and its association with internal H^+ (to form intracellular NH_4^+), followed by a gradual acidification due to slow influx of NH_4^+. Upon removal of external NH_4^+, NH_3 rapidly exits the cells leaving behind H^+, and pH_i falls to a level less than the initial value due to the previous net entry of NH_4^+ (44, 55).

Properties of membrane-based exchange agents which are involved in regulation of pH_i are described below.

Na^+/H^+ Antiport. The Na^+/H^+ antiport is a plasma membrane-associated transporter found in most animal cells (56). It has been implicated in the regulation of pH_i, intracellular Na^+ concentration, and cell volume and may also be involved in the early stages of mitogenesis. The activity of the exchanger may be estimated from the rate of change of pH_i, measured fluorimetrically after loading cells with BCECF, following addition of Na^+ to acid-loaded cells suspended in Na^+-free solution (Fig. 2).

The Na^+/H^+ antiport transports Na^+ and H^+ across the cell membrane with a 1:1 stoichiometry and is therefore electroneutral (57, 58). Although Na^+/H^+ exchange is reversible, physiological gradients of Na^+ and H^+ favor Na^+ influx and H^+ efflux with consequent cytoplasmic alkalinization. The prevailing inward Na^+ gradient is maintained by the Na^+/K^+ ATPase, which therefore indirectly fuels the Na^+/H^+ exchanger. The Na^+/H^+ antiport is not dependent on metabolic energy for its operation (59, 60), but its activity is partially inhibited in ATP-depleted cells (61, 62). ATP may influence Na^+/H^+ exchange activity by phosphorylation of the antiport or of a regulatory protein.

The activity of the Na^+/H^+ antiport depends on Na^+ and H^+ concentration. In general, lowering $[Na^+]_e$ and pH_e leads to progressive inhibition of the antiport, whereas lowering $[Na^+]_i$ and pH_i leads to its stimulation (56, 58). The exchanger becomes inactive at alkaline pH_i. The exchanger appears to have a single binding site for external Na^+ but has a second cytoplasmic H^+-binding site ("modifier site") that allosterically controls its activity (63, 64). Such modification is necessary because thermodynamic considerations predict that if Na^+ and H^+ were distributed across the plasma membrane according to their chemical gradient alone, the steep inward Na^+ gradient would drive the resting pH_i to $pH_i > 8.0$.

Studies of substrate specificity of the Na^+/H^+ antiport show that it can transport Li^+ and with a lower affinity also NH_4^+, but not K^+, Rb^+, Cs^+, Mg^{2+}, Ca^{2+}, or organic cations. The gene encoding the Na^+/H^+ exchanger has been cloned recently (65). The structure of the corresponding protein appears to have 10 transmembrane spanning segments, with a molecular weight of about 100,000 (65). It can also mediate Na^+/Na^+ exchange (56).

The K^+-sparing diuretic amiloride is a weak base which inhibits Na^+/H^+ exchange. Its inhibition is asymmetrical and confined to the extracellular side of the membrane (66). Although amiloride can compete with extracellular Na^+, Franchi *et al.* (67) have isolated 2 types of mutant fibroblasts which overexpress Na^+/H^+ exchange activity and have used these mutants to demonstrate that the binding sites for Na^+ and amiloride are distinct.

At concentrations known to inhibit Na^+/H^+ activity (~100 μM), amiloride has been found to inhibit directly a variety of cellular processes, including several transport systems such as Na^+/K^+ ATPase, Na^+/Ca^{2+} exchange, Na^+/hexose, and Na^+/amino acid transport, several tyrosine and serine kinases, protein synthesis, and the activity of topoisomerase II (reviewed in Ref. 68). A series of amiloride analogues has been synthesized (69). In general, alkyl substitution of the 5-amino group (*e.g.*, dimethylamiloride, ethylisopropylamiloride) greatly enhances the potency of inhibition of Na^+/H^+ exchange (up to 100-fold) (70, 71). The study of inhibition of Na^+/H^+ exchange by one of these compounds has been used to demonstrate the presence of 2 pharmacologically distinct exchange agents in the membrane of porcine kidney cells (72).

Reversible inhibition of Na^+/H^+ exchange can be achieved also by guanidinium or its derivatives [*e.g.*, guanochlor (73)] and by several alkaloids [*e.g.*, quinidine and harmaline (74, 75)]. The antiport is inhibited covalently by compounds such as the carboxyl group-specific dicyclohexylcarbodiimide (76, 77), the histidine-specific diethylpyrocarbonate, and the sulfhydryl group-specific *N*-ethylmaleimide (78). The latter compounds, however, show reduced specificity towards Na^+/H^+ exchange relative to amiloride.

Bicarbonate-dependent Transporters. Bicarbonate-dependent pH_i regulation is carried out by Na^+-coupled and Na^+-independent HCO_3^-/Cl^- exchange mechanisms. These transporters are both sensitive to stilbene derivatives such as DIDS and 4-acetamido-4′-isothiocyanostilbene and insensitive to amiloride. Recent studies showing differential pH dependence (see below) and differential sensitivity to ethacrynic acid and picrylsulfonic acid (79) have demonstrated that these bicarbonate-dependent transporters are distinct from each other. Their activity can be measured by observing changes in pH_i after adding acid-loaded cells containing BCECF to bicarbonate-containing medium, in the presence or absence of Na^+, and in the presence of amiloride or one of its analogues to inhibit Na^+/H^+ exchange (*cf.* Fig. 2).

The Na^+-dependent HCO_3^-/Cl^- exchange agent participates in acid extrusion (44, 80–83). Whereas most cells appear to utilize Na^+/H^+ exchange to regulate pH_i under acid conditions, recent evidence suggests that presence and activity of the Na^+-dependent HCO_3^-/Cl^- exchanger are quite variable among mammalian cell lines (84). The transporter is electroneutral and is believed to exchange extracellular Na^+ and HCO_3^- for intracellular Cl^- and (possibly) H^+, with a stoichiometry of 1 Na^+:1 Cl^-:2 acid/base equivalents (44, 85). Entry of HCO_3^- into the cell allows buffering of H^+ according to the reactions $H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons H_2O + CO_2$, with a net loss of protons as CO_2 diffuses freely out of the cell. The Na^+-dependent HCO_3^-/Cl^- exchanger is quiescent at alkaline pH_i and becomes activated as pH_i falls below a certain threshold (81–83).

The large inward Na^+ gradient is sufficient to fuel Na^+-coupled HCO_3^-/Cl^- exchange, but there is evidence that exchange activity is inhibited by depletion of ATP in some invertebrate cells (86) and in Chinese hamster ovary cells (62).

The cation-independent HCO_3^-/Cl^- exchanger transports anions across the cell membrane with a 1:1 stoichiometry and is therefore electroneutral (87). In most cells, the inward chloride gradient is greater than that of bicarbonate. Because the stoichiometry of the exchange is 1:1, Cl^- is expected to enter the cell in exchange for HCO_3^-, leading to cytoplasmic acidification (88). Accordingly, the HCO_3^-/Cl^- exchanger has been demonstrated to reduce pH_i in a variety of mammalian cells (82, 83), but cells are rarely faced with the problem of alkaline loading under physiological circumstances. HCO_3^-/Cl^- exchange was found to be inhibited at acidic pH_i in several cell types in culture (51, 82, 83) and appears to play at most a

minor role in regulation of pH_i under acidic conditions.

Additional Mechanisms Which Contribute to Regulation of pH_i. Additional membrane-based mechanisms probably contribute to regulation of pH_i in mammalian cells, since some cells are able to survive in nominally bicarbonate-free medium in the absence of Na^+/H^+ exchange. An ATPase-linked proton pump plays the major role of regulating pH_i in yeast, and a related mechanism has been found in some specialized epithelial cells (89).

Lactic acid is the end product of the glycolytic pathway and must be transported out of cells. Since the pK_a of lactic acid is 3.9, it is dissociated within the range of pH that is consistent with cell viability. Diffusion across the cell membrane is therefore believed to be a minor process. Lactate may be transported by the cation-independent anion exchanger but most of the lactate transport appears to take place via a pathway that specifically transports lactate and other monocarboxylic acids such as pyruvate (28, 29, 90, 93). The transport is electroneutral, probably involving a symport with H^+ (92, 93); it therefore contributes to removal of protons, and in rat hepatocytes it accounted for about 18% of Na^+-independent proton efflux (54). The pathway is inhibited competitively by pyruvate and other substituted monocarboxylic acids and their analogues (94, 95), by the compounds α-cyano-3 (or-4)-hydroxycinnamate, by thiol group reagents such as mersalyl (28, 90), and by bioflavinoids such as quercetin (96, 97).

Influence of pH_i Regulation on Mitogenesis and Tumor Growth

Role of Na^+/H^+ Exchange in Mitogenesis. The first direct demonstration that cell proliferation could be induced by an increase in pH_i, mediated by Na^+/H^+ exchange, came from studies on the activation of the sea urchin egg (98). Since then, numerous studies have presented evidence which suggests that activation of the antiport and/or the consequent cytoplasmic alkalinization can be important precursors of cell proliferation. This evidence has been reviewed recently by Grinstein *et al.* (68) and is presented briefly below. (*a*) Many known mitogens and comitogens, including growth factors, mitogenic lectins, the *ras* oncogene product (M_r 21,000 protein), and tumor-promoting phorbol esters, have been shown to cause rapid activation of Na^+/H^+ exchange without prior cytoplasmic acidification. This usually occurs, however, in medium that is free of bicarbonate. Activation of the Na^+/H^+ antiport under such conditions results in an elevation of pH_i by 0.15–0.30 unit above the resting pH_i of ~7.0–7.2 (99–102). The above stimuli appear to cause an elevation of the threshold pH_i controlled by the modifier site, leading to an alkaline shift in the pH_i sensitivity of the antiport. (*b*) Amiloride and its analogues have been shown to inhibit egg activation (cell division) in the sea urchin (98) and growth factor-induced DNA synthesis in several mammalian cell types (*e.g.*, 70, 96). Inhibition of DNA synthesis can also be observed, however, in the absence of extracellular sodium. In hamster lung fibroblasts, L'Allemain *et al.* (70) have demonstrated that in bicarbonate-free medium with low $[Na^+]_e$, growth factor-induced DNA synthesis of G_0-arrested cells was inhibited by amiloride analogues with the same rank order as that for inhibition of the Na^+/H^+ antiport. (*c*) In some cells, it has been claimed that DNA synthesis and cell proliferation can be induced by cytoplasmic alkalinization in the absence of mitogens (97). In addition, transfection of fibroblasts with the gene coding for the yeast H^+-ATPase pump led to elevation of pH_i and to cell transformation, two phenomena that were not observed upon transfection with a defective H^+-ATPase gene (103). Thus elevation of pH_i (but not necessarily activation of the Na^+/H^+ antiport) may be a primary mechanism which initiates proliferation of some types of cell. In contrast to the above evidence for the involvement of the Na^+/H^+ antiport (or cellular alkalinization) in mitogenesis, recent studies have demonstrated that activation of the antiport and elevation of pH_i are probably not sufficient and may not even be necessary for proliferation of many types of cell. A brief summary of this evidence is presented below (see Ref. 68 for review). (*a*) A variety of agents and conditions which activate the Na^+/H^+ antiport (*e.g.*, several hormones, osmotic shrinking) are not mitogenic (64, 104). Moreover, cytoplasmic alkalinization alone does not lead to stimulation of proliferation in most cells (*e.g.*, Ref. 15). (*b*) In some cells, mitogen-induced cell proliferation proceeds normally in the absence of prior cytoplasmic alkalinization (106, 107). (*c*) Amiloride and its analogues are not always inhibitory to cell proliferation (107–108) and the inhibitory effects that are observed may be due to nonspecific effects of the drugs, such as direct inhibition of protein or DNA synthesis. In lymphocytes (106, 109) and in a human breast cancer cell line (109), exposure to amiloride analogues in the absence of bicarbonate, at doses which eliminated Na^+/H^+ exchange, had no effects on mitogen-stimulated expression of the oncogenes c-*fos* and c-*myc*, on DNA synthesis, or on cell growth. (*d*) In the presence of bicarbonate, arginine vasopression was shown to stimulate both Na^+-dependent and -independent HCO_3^-/Cl^- exchange, as well as Na^+/H^+ exchange, leading to a net decrease in pH_i in growth-stimulated renal mesangial cells (110). (*e*) A reduced growth rate of Na^+/H^+ exchange-deficient mutants as compared to their respective parental cells was observed only at physiological or acid pH_e in the absence of bicarbonate (111–113).

Taken together, the evidence presented above suggests that in most mammalian cells the Na^+/H^+ antiport and cytoplasmic alkalinization are not necessary to stimulate cellular proliferation. The antiport may be essential for cell proliferation only under restricted conditions such as those in which bicarbonate levels are low and the Na^+-coupled HCO_3^-/Cl^- exchanger is inoperative. Such conditions might occur in acidic regions of tumors.

Regulation of pH_i and Tumor Growth. Under the acidic conditions that occur in solid tumors, regulation of pH_i may play an important role in maintaining the viability of tumor cells. Acid extrusion in most mammalian cells is carried out via the Na^+/H^+ antiport and the Na^+-dependent HCO_3^-/Cl^- exchanger, but according to the Henderson-Hasselbalch equation, HCO_3^- concentration decreases with decreasing pH. Thus, in cells surrounded by an acidic environment in which bicarbonate concentration is reduced, the Na^+/H^+ antiport may have an important role in controlling pH_i.

To study the importance of pH_i regulation for tumor formation and growth, the activity of the pH_i-regulating ion exchange mechanisms may be eliminated either with specific inhibitors or by a mutation of the gene(s) encoding the transporter protein. A strategy for the isolation of Na^+/H^+ exchange-deficient mutants was developed by Pouyssegur *et al.* (111) and is illustrated in Fig. 4. Cells are exposed to a mutagen, loaded with Li^+, and then exposed to a very low pH_e (5.5) in the absence of external Na^+ or Li^+. Because the antiport is reversible and can also transport Li^+, this leads to Li^+ efflux and H^+ influx, resulting in severe cytoplasmic acidification and death of cells possessing, but not of cells lacking, the antiport. Thus far, Na^+H^+-deficient mutants have been isolated from Chinese hamster lung fibroblasts (111) pig kidney epithelial cells (112), mouse L cells

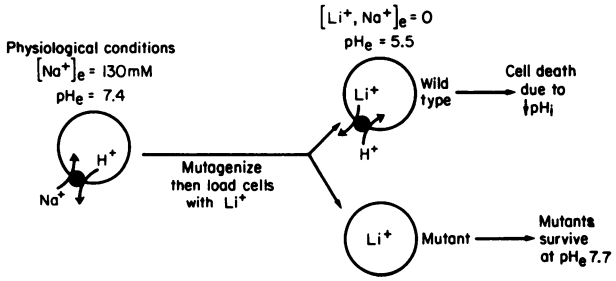

Fig. 4. Method used to select variant cells which lack Na^+/H^+ exchange activity. Cells are exposed to a mutagen and then loaded with Li^+. When placed in choline chloride solution at pH_e ~5.5, wild type cells exchange Li^+ for H^+ and die from cytoplasmic acidification. This occurs because the Na^+/H^+ antiport is reversible and recognizes Li^+ as an alternative substrate to Na^+. Variant cells which lack Na^+/H^+ exchange activity are selected and will grow in alkaline medium [modified from the report of Pouyssegur *et al.* (111)].

Table 1 *Growth of xenografts from MGH-U1 human bladder cancer cells, and from their sodium-proton exchange-deficient (HSPD) sublines*

	Tumor incidence (%)
MGH-U1 (wild type)	34/35 (97)
HSPD-1	6/21 (29)
HSPD-2	0/23 (0)
HSPD-2 revertant	8/8 (100)
Exchange proficient MGH-U1 cells surviving selection procedure	15/16 (94)

(114), Chinese hamster ovary cells,[5] and human bladder carcinoma MGH-U1 (EJ) cells (113).

Na^+/H^+-deficient cells were unable to grow in tissue culture at pH_e <7.0–7.2 in media nominally free of bicarbonate, whereas their respective parental (Na^+/H^+ exchange competent) cells grew well at pH_e 0.2–0.4 unit lower (111–113); this differential acid sensitivity was abolished in the presence of bicarbonate. Thus, in an environment of reduced bicarbonate concentration *in vivo*, lack of Na^+/H^+ antiport activity might impair tumor growth.

Assessment of tumor growth of Na^+/H^+-deficient cells has been carried out in two cell lines: Chinese hamster lung fibroblasts (115, 116); and the human bladder carcinoma MGH-U1 cells (113). Only the latter is a cell line derived from malignant cells, although the hamster fibroblasts (CCL39) form tumors in athymic nude mice after a long latency period. Inoculation s.c. of Na^+/H^+-deficient mutants isolated from both CCL39 and MGH-U1 cells into immunodeficient mice led to either a complete loss or severe retardation of tumor growth relative to that observed after implantation of their respective parental cells (Refs. 113 and 116; Table 1). Loss of tumor-forming ability of variant MGH-U1 cells could not be attributed to pretreatment with a mutagen, because cells which survived the same isolation procedure but which did not lose Na^+/H^+ exchange activity retained their tumorigenic capacity (113). In addition, spontaneous revertant cells, isolated from the Na^+/H^+ exchange-deficient mutants, regained both Na^+/H^+ exchange activity and tumorigenicity. Moreover, cells derived from the few slowly growing tumors which developed after implantation of Na^+/H^+ exchange-deficient MGH-U1 variants have regained antiport activity (113). Taken together, these results suggest that the presence of the antiport is important, and may be even required, for growth of MGH-U1 tumors.

In the hamster fibroblasts, although initial tumor incidence was lower in the antiport-deficient (PS120) cells relative to the parental CCL39 cells, reimplanting cells excised from PS120 tumors, which retained their Na^+/H^+-deficient phenotype, led to a substantial recovery of tumorigenic capacity (115, 116). These results suggest that in hamster fibroblasts, the initial acquisition or expression of the tumorigenic phenotype is more efficient in the presence of the Na^+/H^+ antiport. In support of this view, Perona and Serrano (103) have shown recently that transfection of fibroblasts with the yeast H^+-ATPase gene led to elevation of cytoplasmic pH, to cell transformation, and to increased tumorigenic capacity.

A plausible (albeit speculative) explanation for the dependency of MGH-U1 cells, but not of Chinese hamster lung fibroblasts, on Na^+/H^+ exchange for tumor growth may relate to differences in the tumor microenvironment that result from differences in growth rate. Parental MGH-U1 cells form rapidly growing xenografts which become palpable at 2 weeks after transplantation and which grow exponentially with a doubling time of about 1 week (17). They form regions of necrosis and probably develop regions of hypoxia and acidity at an early stage of growth. Survival of constituent tumor cells may then be dependent on the presence of Na^+/H^+ exchange, and tumor growth would continue from exchange-deficient mutants only if revertant cells are selected with Na^+/H^+ exchange proficiency. In contrast, Chinese hamster lung fibroblasts form slowly growing tumors in nude mice with a latency period of 4–6 weeks. Although the exchange-proficient cells selected from primary tumors grow more rapidly on reimplantation, the Na^+/H^+-deficient cells which are derived from initial tumors continue to produce slowly growing xenografts with a latency of 3–6 weeks and doubling times of 1–3 weeks (116). This slower rate of growth may allow the development of an adequate vasculature from the host animal, with better nutrition and less tendency to develop acidosis. Thus the tumor-forming Chinese hamster lung fibroblasts may be less dependent for their survival *in vivo* on mechanisms which regulate pH_i.

An alternative, albeit less specific, method to assess the importance of the Na^+/H^+ antiport for tumor growth is by use of Na^+/H^+ exchange inhibitors such as amiloride or its analogues. Indeed, Sparks *et al.* (118) have demonstrated significant suppression of growth of the H6 hepatoma and DMA/J mammary carcinoma in mice treated repeatedly with amiloride, as compared to untreated animals. *In vitro*, clonogenic capacity of both CHO and MGH-U1 cells was impaired following acute exposure of acid-loaded cells to amiloride at low external pH (119). Amiloride and its more potent analogues have been shown to have several effects on cellular metabolism, and these compounds should be tested for their effects on tumor growth in animals.

Participation of the Na^+-coupled HCO_3^-/Cl^- exchange mechanism in pH_i regulation in cells located in acidic regions of tumors is likely to be dependent on the concentration of bicarbonate and on the activity of this ion exchange mechanism. There have been no Na^+-coupled HCO_3^-/Cl^- exchange-deficient mutants isolated to date, so that studies of tumorigenicity comparable to those performed with Na^+/H^+-deficient mutants have not been carried out.

Tumor pH and Treatment

Many cellular processes depend on pH. These include synthesis of macromolecules and cell proliferation, transport of metabolites and drugs, and the activity of enzymes. It is to be expected, therefore, that the effects of therapeutic agents may depend on intracellular or extracellular pH (or both). Moreover, the low pH in tumors may contribute to cell death even in the absence of therapy. The influence of pH on cell viability and

[5] L. Siminovitch, personal communication.

on the activity of therapeutic agents is reviewed below.

Low pH and Cell Death in Untreated Tumors. In tissue culture, most mammalian cells will not proliferate in medium at a pH lower than about 6.6 (120, 121). Cell viability as assessed by colony formation also decreases after chronic exposure to low pH, but there is little loss of viability after short exposure (<6 h) under aerobic conditions to medium at pH_e as low as 6.0 (120, 122). It is expected, however, that hypoxia and acidosis may coexist in some regions of solid tumors, and when these conditions were simulated in culture we found a very rapid loss of colony-forming ability in two mammalian cell lines (Ref. 122; Fig. 5). Investigation of cell metabolism under these conditions showed that cells had a marked reduction in ATP levels, presumably because of inhibition of glycolysis at low pH and of respiration under hypoxic conditions (122). Whereas cells incubated at low pH_e under aerobic conditions were able to maintain pH_i about 0.25 unit higher than pH_e, this gradient tended to be lost under hypoxic conditions, probably as a result of inhibition of the Na^+/H^+ and Na^+-dependent HCO_3^-/Cl^- exchange mechanisms which regulate pH_i under acidic conditions (62, 123, 124). Inhibition of the exchangers may have resulted from ATP deprivation as reported for other cells (55, 61, 125, 126).

The causes of cell death within solid tumors are poorly understood. The above data are provocative that low pH may have a major role in contributing to this process. The decreased or absent tumor formation from mutant cells lacking Na^+/H^+ exchange (see above) is consistent with this hypothesis.

Ionizing Radiation. Cell survival after ionizing radiation has been assessed at physiological and at low pH for several mammalian cell lines (127–130). The results of these experiments have been consistent in demonstrating increased radiation resistance at reduced pH_e, although the effect on radiation sensitivity is much less than that due to hypoxia. In two of the above studies the major effect of low pH_e was to increase the width of the shoulder region of the cell survival curve (127, 128), implying greater capacity for DNA repair under acidic conditions. Such an effect would be magnified when radiation is delivered in multiple small fractions, as in human tumor radiotherapy. In contrast, the studies of Freeman *et al.* (129, 131) using CHO cells show little or no effect of low pH in increasing the width of the shoulder region of the survival curve.

The time at which cells are rendered acidic in relation to delivery of radiation influences their survival. Exposure of CHO cells to acid conditions after irradiation gave increased survival as compared to acid exposure before or during radiation (132). The probable mechanism is that acid conditions inhibit the fixation of potentially lethal radiation damage (131).

The above results were obtained on exponentially growing cells that were irradiated under aerobic conditions. In solid tumors, many cells may be both acidic and hypoxic, and under these conditions cell proliferation is inhibited. Freeman and Sierra (130) found that low pH did not increase the radioresistance of unfed plateau phase cells in culture, and these cells may provide a better model for slowly proliferating cells in acidic and nutrient-deprived regions of solid tumors. It seems unlikely, therefore, that acidity adds to radioresistance in hypoxic tumor regions.

Hyperthermia. Hyperthermia is an experimental treatment for human cancer, and research into the modality has been stimulated by the consistent evidence that low pH_e exerts a major effect in sensitizing cultured cells to heat (2, 133–137). Since hypoxia at normal pH_e has minimal effects on cellular sensitivity to hyperthermia, it seems feasible that hyperthermia could kill selectively acidic and hypoxic cells in solid tumors (because of acidosis) and hence complement the effects of ionizing radiation which tend to spare such cells (because of hypoxia).

The influence of pH_e on survival of cultured cells under different conditions of heating is illustrated in Fig. 6. Although there are differences among cell lines, these data demonstrate that low pH_e usually causes greater sensitization to heat at lower temperatures (134, 135, 137), and that quite dramatic effects can be achieved by a relatively modest decrease in pH_e, within the range of 6.5–7.0, that is found commonly in solid tumors. Recent evidence suggests, however, that cells which are adapted to grow at low pH_e tend to lose their differential thermal sensitivity as a function of pH_e (138, 139). Response to heat appears to be a function of pH_i rather than pH_e (140), and loss of heat sensitization in cells adapted to low pH_e may result from regulation of pH_i to physiological levels (139). The effects of hyperthermia at low pH_e were strongly enhanced in the presence of CCCP which lowers pH_i (141), and one might

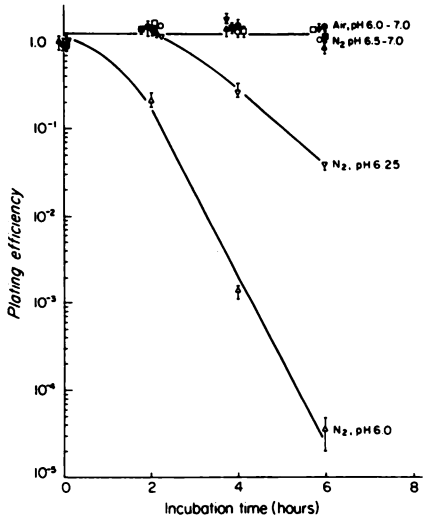

Fig. 5. Plating efficiency of Chinese hamster ovary cells incubated in air (*closed symbols*) or hypoxia (N_2, *open symbols*) for up to 6 h at pH_e 6.0 (▲, △), 6.25 (▼, ▽), 6.5 (■, □), or 7.0 (●, ○). Mean and range for triplicate plates are plotted, and data are representative of more than 10 experiments. Qualitatively similar results have been obtained with other cell lines [modified from Rotin *et al.* (122)].

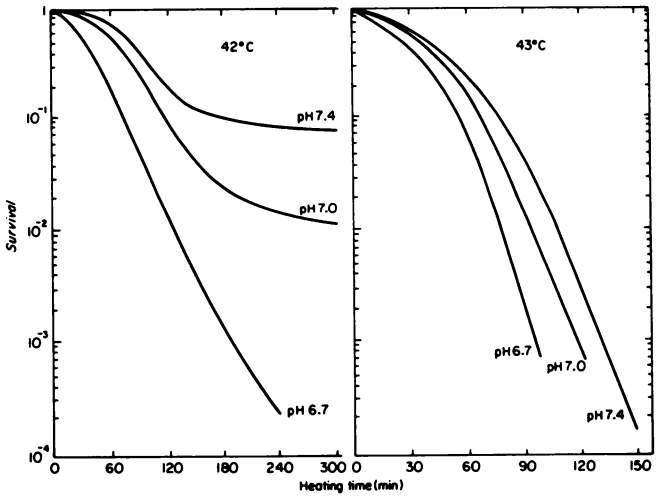

Fig. 6. Survival of Chinese hamster ovary cells following heating for various times at 42°C or 43°C while the cells were in growth medium at different pH [modified from Gerweck (135)].

expect persistence of this effect in cells adapted to low pH_e. Amiloride has also been found to increase thermal sensitivity, perhaps by impairing pH_i regulation, although this effect is observed both at physiological and at acidic pH_e (142).

A major limitation to the application of heat for cancer treatment is the development of thermotolerance, whereby an initial heat treatment leads to the induction of resistance to subsequent heat treatments delivered within the next 3–5 days. Maintenance of cells at low pH_e after heating was found to inhibit the development of thermotolerance (143, 144), but this inhibition of thermotolerance at low pH_e was not observed in cells that were adapted to such conditions (138). Thus both single and fractionated heat treatments would be expected to be selectively toxic only to cells which have become acutely acidic within solid tumors, and these selective effects of heat would be lost as the cells became chronically adapted to low pH_e. Agents which cause acute acidification of cells in the presence of low pH_e might play a therapeutic role in the treatment of solid tumors by hyperthermia.

The mechanism(s) by which heat treatment kills cells is unknown, although damage to cell membranes and protein denaturation are believed to play a primary role (145). Recent studies of pH_i during heating of CHO cells to 42°C or 45°C at pH_e 6.6 have shown unexpected initial increases in pH_i, although subsequent intracellular acidification was also observed (146, 147). Thus there appears to be no immediate breakdown in mechanisms regulating pH_i, although the effects of heat on specific membrane exchange mechanisms have not been determined.

Treatment with heat leads to a rapid fall in pH_e in many solid tumors. This effect appears to be due to a marked decrease in blood flow because of vasoconstriction and coagulation necrosis (2, 3, 148, 149). Hyperthermia often stimulates blood flow initially in normal tissues unless the temperature exceeds ~44°C (150, 151) and this effect may contribute to selective killing of cells in tumors, as compared to those in normal tissues, during subsequent heat treatment.

Chemotherapy. Anticancer drugs must be transported into cells, by either active transport or passive diffusion, and frequently undergo intracellular metabolism. Since all of these processes, as well as the metabolic pathways that are inhibited, depend on pH, it is expected that the cytotoxic activity of anticancer drugs may depend on both pH_e and pH_i. In particular, where uptake of drugs which contain acidic or basic charged groups is by passive diffusion, transport will be enhanced at values of pH_e that favor the nonionized form of the compound.

Data on the activity of commonly used anticancer drugs as a function of varying pH_e in cell culture are summarized in Table 2. Additional data indicate that low pH_e enhances the interaction of several drugs (*e.g.*, bischloroethylinitrosourea, bleomycin) when used with hyperthermia, and this effect is much greater than the enhancement of either agent used alone (138, 154).

The influence of pH_e on the *in vivo* activity of drugs is likely to be complex since the more acidic regions of tumors are probably situated distal from functional blood vessels. Thus the net effect of anticancer drugs against cells in acidic regions of tumors will depend also on their penetration into tissue from the blood vessels through which they are delivered. Penetration is known to be poor for some drugs such as doxorubicin (162, 163).

There have been attempts to use selectively drugs whose uptake into cells is favored at low pH_e. The alkylating agent triethylenemelamine was used in an attempt to selectively kill cells in a solid tumor (presumed hypoxic and acidic) that survived ionizing radiation; although the drug caused delay to recurrence after radiation it did not enhance the probability of cure (164). Others have shown some increased activity of triethylenemelamine and other alkylating agents for experimental tumors after treating animals with glucose (165, 166). This effect may be due to a fall in tumor pH but could also be influenced by changes in tumor metabolism after treatment with glucose.

It is apparent that data relating to the effects of pH_e on the activity of anticancer drugs are incomplete, and most of the available data summarized in Table 2 have been obtained (for each drug) on only one or two cell lines. The use of fluorescent dyes, such as Hoechst 33342, which allow the separation of cells at different distances from functional blood vessels in tumors by flow cytometry, allows study of drug effects in different tumor regions (169). Improvements in technology might allow these methods to be combined with the use of fluorescent probes which indicate pH_i *in vivo* (50) and would facilitate study of the relationship between drug activity and pH_i in tumors.

New Approaches to Therapy. Acidic pH in tumors may enhance the effectiveness of conventional therapeutic modalities. Acidity might also allow the development of new approaches targeted toward the cellular mechanisms that regulate pH_i. Support for the feasibility of this approach derives from the observation that mutant cells which lack Na^+/H^+ exchange have either absent or reduced ability to generate tumors (113, 116) and from the demonstration of (acid) pH-dependent cell killing by agents which impair regulation of pH_i (119, 127).

Ionophores are compounds which allow transport of ions across the cell membrane and have relative specificity for different ionic species (47, 67). The ionophore CCCP allows protons to cross the membrane according to their electrochemical gradient. Since the membrane potential of cells is negative inside, CCCP causes influx of protons and a decrease of pH_i. Nigericin allows electrically neutral exchange of K^+ for H^+, and since there is a large outwardly-directed gradient of K^+ (maintained by Na^+/K^+ ATPase), the ionophore causes rapid intracellular acidification. The rapid fall in pH_i induced by these ionophores is illustrated in Figs. 2 and 7, using BCECF and fluorimetry. Both of these compounds cause toxicity that is strongly dependent on pH_e as illustrated in Fig. 8; the ionophores cause little or no toxicity above pH_e 6.6, with a rapid and dose-dependent fall in cell survival as pH_e is reduced below this value (119, 127).[6] Drugs such as amiloride (which inhibits Na^+/H^+ exchange) or DIDS (which inhibits Na^+-coupled and Na^+-independent HCO_3^-/Cl^- exchange) did not have major toxic effects when used alone but enhanced the killing of cells that were acidified with nigericin or CCCP. The drug combination of amiloride plus DIDS applied to acid-loaded cells led to marked cell killing at pH_e 6.9 or below, well within the range observed in many solid tumors (119).

Other drugs which might be expected to cause pH-dependent cytotoxicity include inhibitors of lactate transport. Lactic acid is dissociated at any pH_i that is consistent with cell survival, and lactate is transported in a 1:1 symport with H^+. Thus inhibition of lactate transport may lead to intracellular acidification, and this effect will be most pronounced in cells that have a high rate of glycolysis, as may occur in tumors. Lactate transport is inhibited by bioflavinoids such as quercetin (168–

[6] K. Newell and I. F. Tannock, unpublished data.

Table 2 *Influence of pH_e on the activity of anticancer drugs in cell culture*

Drug	Effect of low pH on drug activity	Mechanism	Ref.
Alkylating agents			
Chlorambucil	Increased	Increased uptake	Mikkelsen and Wallach (152)
Thiotepa	Increased	NS[a]	Euler *et al.* (153)
BCNU	Minimal		Hahn and Shiu (140, 154)
CCNU	Decreased	NS	Kwok and Twentyman (155)
Mitomycin C	Increased	Increased drug activation	Kennedy *et al.* (156)
Doxorubicin	Decreased	Decreased uptake	Born and Eicholtz-Wirth (157) Hindenburg *et al.* (158)
Bleomycin	Minimal		Hahn and Shiu (138, 154) Urano *et al.* (159)
Cisplatin	Increased	NS	Herman *et al.* (160)
Vinblastine	Decreased	Decreased uptake	Ferguson *et al.* (161)
Vincristine	Decreased	Decreased uptake	Ferguson *et al.* (161)
Methotrexate	Minimal		Hahn and Shiu (154)

[a] NS, not stated; BCNU, bischloroethylnitrosourea; CCNU, cyclohexylchloroethylnitrosourea.

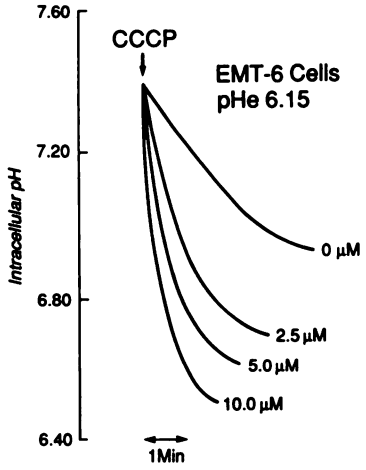

Fig. 7. Acidification of EMT-6 cells following exposure to different concentrations of CCCP in medium at pH_e 6.15. pH_i was measured by the BCECF method. Qualitatively similar effects are obtained with other cell lines, or after exposure of cells to nigericin.[6]

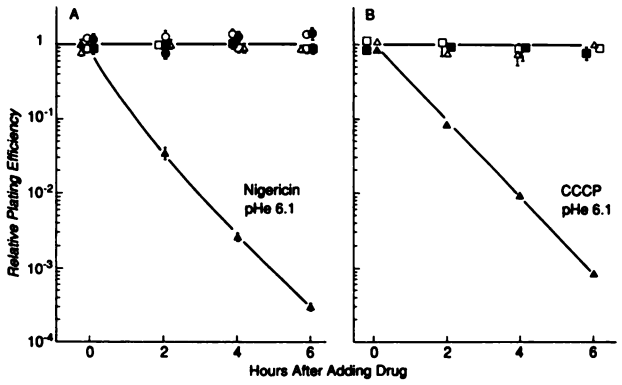

Fig. 8. Relative plating efficiency of MGH-U1 human bladder cancer cells exposed to the ionophores (*A*) nigericin (0.25 μl/ml) and (*B*) CCCP (15 μM) at pH_e 6.1 (▲) or at pH_e 7.0–7.3 (■). Conditions leading to 100% cell survival included exposure to pH_e 7.0–7.3 (□), 6.5 (○), or 6.1 (△) without drug, drug exposure to either drug at physiological pH_e (■), and exposure to nigericin at pH_e 6.5 (●). *A* adapted from Rotin et al. (113); for *B* see Footnote 6.

170) but these compounds are inactivated by serum. Other inhibitors include derivatives of lactic acid itself (94, 95) and α-cyano-4 (or -3)-hydroxycinnamate (28, 90). The cytotoxicity of these compounds, and its dependence on pH_e, have not yet been tested.

All of the compounds described in the present section have biological effects in addition to those which might lead to pH-dependent cytotoxicity. It seems unlikely therefore that these pilot compounds will be sufficiently specific to cause pH-dependent cytotoxicity *in vivo* with adequate normal tissue tolerance. Moreover, it will be essential for effective compounds to have good penetration in tissue to acidic microregions of tumors, and their therapeutic potential may be maximal when used with agents that eradicate well nourished cells proximal to tumors blood vessels (*e.g.*, radiation or doxorubicin).

There are few properties of tumors which offer the prospect of specific cell killing. It seems important therefore to try to take advantage of physiological differences which exist and to develop compounds that maintain the strong pH-dependent cytotoxicity that can now be observed in cell culture and which have properties which may allow them to be tested *in vivo*. A mechanistic approach to drug development seems more likely to succeed than the random testing of new compounds. Also, tumor models used for evaluation of new drugs should contain subpopulations of cells which reflect the heterogeneity that is present in the microenvironment of solid tumors in humans.

Summary

Measurement of pH in tissue has shown that the microenvironment in tumors is generally more acidic than in normal tissues. Major mechanisms which lead to tumor acidity probably include the production of lactic acid and hydrolysis of ATP in hypoxic regions of tumors. Further reduction in pH may be achieved in some tumors by administration of glucose (±insulin) and by drugs such as hydralazine which modify the relative blood flow to tumors and normal tissues.

Cells have evolved mechanisms for regulating their intracellular pH. The amiloride-sensitive Na^+/H^+ antiport and the DIDS-sensitive Na^+-dependent HCO_3^-/Cl^- exchanger appear to be the major mechanisms for regulating pH_i under conditions of acid loading, although additional mechanisms may contribute to acid extrusion.

Mitogen-induced initiation of proliferation in some cells is preceded by cytoplasmic alkalinization, usually triggered by stimulation of Na^+/H^+ exchange; proliferation of other cells can be induced without prior alkalinization. Mutant cells which lack Na^+/H^+ exchange activity have reduced or absent ability to generate solid tumors; a plausible explanation is the failure of such mutant cells to withstand acidic conditions that are generated during tumor growth.

Studies in tissue culture have demonstrated that the combination of hypoxia and acid pH_e is toxic to mammalian cells, whereas short exposures to either factor alone are not very toxic. This interaction may contribute to cell death and necrosis in solid tumors. Acidic pH may influence the outcome of tumor therapy. There are rather small effects of pH_e on the response

of cells to ionizing radiation but acute exposure to acid pH_e causes a marked increase in response to hyperthermia; this effect is decreased in cells that are adapted to low pH_e. Acidity may have varying effects on the response of cells to conventional anticancer drugs. Ionophores such as nigericin or CCCP cause acid loading of cells in culture and are toxic only at low pH_e; this toxicity is enhanced by agents such as amiloride or DIDS which impair mechanisms involved in regulation of pH_i. It is suggested that acid conditions in tumors might allow the development of new and relatively specific types of therapy which are directed against mechanisms which regulate pH_i under acid conditions.

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