Heterogeneity in pHi and the Selection for Variants with Increased and Decreased Na^+-dependent Cl^-/HCO_3^- Exchanger Activity on the Basis of pHi

by

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Abstract

The Na^+-dependent Cl^-/HCO_3^- exchanger and the Na^+/H^+ antiport are known to regulate the intracellular pH (pHi) of tumor cells, existing in acidic extracellular pH (pHe) environments. The influence of exchanger activity on the relationship between pHe and pHi, and the distribution of pHi within populations of three cell lines was assessed with flow cytometry. At exposure to various levels of pHe, the pHi ranged from 6.8-7.3. Within this range pHi was maintained at a higher level with Na^+-dependent Cl^-/HCO_3^- exchange than with Na^+/H^+ counter-transport. The distribution in pHi that was also observed reflected true biological variation in pHi. On the basis of different levels of pHi, a flow cytometric method was developed to select for variants with increased and decreased levels of Na^+-dependent Cl^-/HCO_3^- exchanger function. It was found that this method was not useful for the derivation of the desired variants. Suggestions for further study have also been outlined.

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Chapter 1

Introduction

1.1 Introduction

Measurements of the pH in the extracellular environment (pHe) of tumors have shown that on average, tumor pHe tends to be about 0.4 pH units less than that of normal tissue. However, techniques that measure internal cellular pH (pHi) demonstrate that it is close to physiological. (Warburg, 1956, Wike-Hooley *et al.*, 1984, Vaupel *et al.*, 1989) These results suggest that tumor cells are regulating their internal pH, since cells can survive only if their cytosolic pH is maintained within a narrow range. (Wike-Hooley *et al.*, '84, Musgrove and Hedley, 1990) In this chapter I will discuss the causes of tumor acidity and the mechanisms that tumor cells are known to utilize in order to regulate pHi.

1.2 Measurements of pHe and pHi in Tumors and Normal Tissue

1.2.1 Extracellular pH

Comparisons of interstitial or extracellular pH (pHe) in tumors and normal tissue have been made in humans and various animals. Measurements of pHe in normal tissue, usually of the subcutis and muscle, indicate a median of approximately 7.5. (Wike-Hooley *et al.*,1984, Thistlethwaite *et al.*, 1985, Vaupel *et al.*,1989) Values of pHe in tumors, in contrast, have been measured to be more acidic than normal tissue - with a median of approximately 7.1. The range of pHe values varied within and between different types of tissue sources studied. In tumors, the pH range was especially broad - 5.8-7.7, as compared to normal tissue (7.0-8.1). This is probably due to

additional factors such as tumor size, histology, and vasculature. Using transparent micropore chambers embedded in tumors that were implanted in rats, Jain *et al.* (1984) observed that interstitial pH (pHe) decreased as tumors increased in size. Furthermore, Koutcher *et al.* (1987) found that when tumor size decreased after irradiation, there was a subsequent increase in tumor pHe. Very necrotic tumors have been found to possess alkaline pHe, whereas large ulcerating tumors were more acidic than the median pHe of non-ulcerated tumors. (0.6 - 0.8 pH units lower) (Vaupel *et al.*, 1981) Using microelectrodes Carlsson and Acker (1988) observed that the pHe in spheroids decreased with increasing depth into the spheroid. Martin and Jain (1994) implanted tumors into transparent chambers in the ears of rabbits and observed a decrease in interstitial pH in tumors as a function of distance from blood vessels (0.13 pH units over 50μm distance) The above results show that the extracellular environment of tumor cells is generally more acidic than that of normal tissue.

1.2.2 Intracellular pH

The pH of the internal milieu of cells from tumors and normal tissues has also been studied. Measurements of normal tissue pHi in skeletal muscle, heart, skin, and brain indicate a mean value of 7.2; ranging from 6.9-7.3. (Vaupel *et al.*, 1989) Values of the pHi in tumors of different histological types appear to be similar to those in normal tissue (mean 7.2, range 6.9-7.4), although in brain tumors pHi has been found to be 0.1-0.2 pH units above normal brain values. (Arnold *et al.*, 1985, Vaupel *et al.*, 1989)

1.3 Causes of Tumor Acidity

Tumor acidity has been of interest because of the potential to exploit this condition in tumor-selective therapy. Low environmental pH has been observed to inhibit cell proliferation, survival, and metabolic activity. (Eagle 1973, Wike-Hooley *et al.*, 1984) Sahler *et al.* (1969) observed that incubation of Erhlich Ascites tumors with glucose induced low pHe and led to the loss of transplantability and inhibition of subsequent tumor growth in mice. More recent studies have shown increased toxicity when tumor cells are exposed to agents which can acidify the cytoplasm of cells (such as nigericin and CCCP) at low pHe. (Newell and Tannock, 1989, Yamagata and Tannock, 1996) For example, Yamagata and Tannock observed killing of cells after prolonged exposure of tumors to nigericin via perfusion pumps. Furthermore, when these pH acidifying agents were combined with drugs that interfered with the ability of cells to regulate pHi (eg. EIPA to block Na^+/H^+ antiport function, DIDS to prevent Na^+-dependent Cl^-/HCO_3^- exchange activity), cell killing was even more pronounced at low pHe. (Rotin *et al.*, 1987, Newell and Tannock, 1989, Maidorn *et al.*, 1992)

Tumor acidity may arise from an imbalance in the relationship between the accumulation of metabolic acids (CO_2 and lactic acid) by tumor cells, and their poor removal by insufficient vasculature. (Tannock, 1968, Sutherland, 1986, Newell and Tannock, 1989) Tumor cells, or surrounding normal cells such as mast cells or macrophages in response to unknown tumor derived factors, release endothelial growth factors (eg. bFGF, VEGF). These growth factors are known to act on the endothelial cells of capillaries and induce proliferation and growth of blood

vessels into tumors. (Folkman and Klagsburn, 1987, Folkman and Shing, 1992). Despite the development of further vasculature within a tumor, the rate is often inadequate to compensate for the speed of tumor growth because the proliferative rate of the vasculature endothelium is slower than that of tumor cells. (Denekamp and Hobson, 1982) These blood vessels also possess structural abnormalities in the vessel walls, and have abnormal architecture, and density; (Vaupel *et al*,. 1989) resulting in the failure to deliver necessary nutrients such as oxygen, and the insufficient removal of potentially harmful catabolites. As a result, the acidic products of metabolism accumulate in the interstitial environment of tumors. (Vaupel *et al.*, 1989)

Rapid tumor growth compounded with poor blood supply results in gradients of nutrients and catabolites. Kallinowski *et al.* (1988) observed in human breast cancer xenografts in nude rats that the metabolism of cells was dependent on the efficiency of the vasculature (ie. blood flow) to supply necessary nutrients Consequently, there is limited diffusion of necessary nutrients such as oxygen and glucose into tumors; and their concentration decreases with increasing distance from the blood supply. (Jahde and Rajewsky, 1982, Kallinowski *et al.*, 1988) It has been shown that the combination of hypoxia and low pHe is toxic to cells in culture, which may contribute to cell death and necrosis in tumors. (Rotin *et al.*, 1986) In addition, the limited penetration of nutrients and oxygen to tumor cells further from the blood supply may be due to the consumption of these nutrients by cells closer to the blood vessels.

Tumor cells utilize glycolysis for energy metabolism and produce lactic acid, even under aerobic conditions. (Warburg, 1956) Cells in hypoxic regions, with little or no access to oxygen, are

constrained to the anaerobic pathway, producing large amounts of the main byproduct - lactic acid, which dissociates to lactate and H^+. (Fig.1.1) (Hochachka and Mommsen., 1983) It has been observed that tumor pHe and pHi decreased after intraperitoneal injections of glucose. (Thistlethwaite *et al.*, 1987) The injection of glucose is thought to result in an increase in glycolysis and lactic acid production.

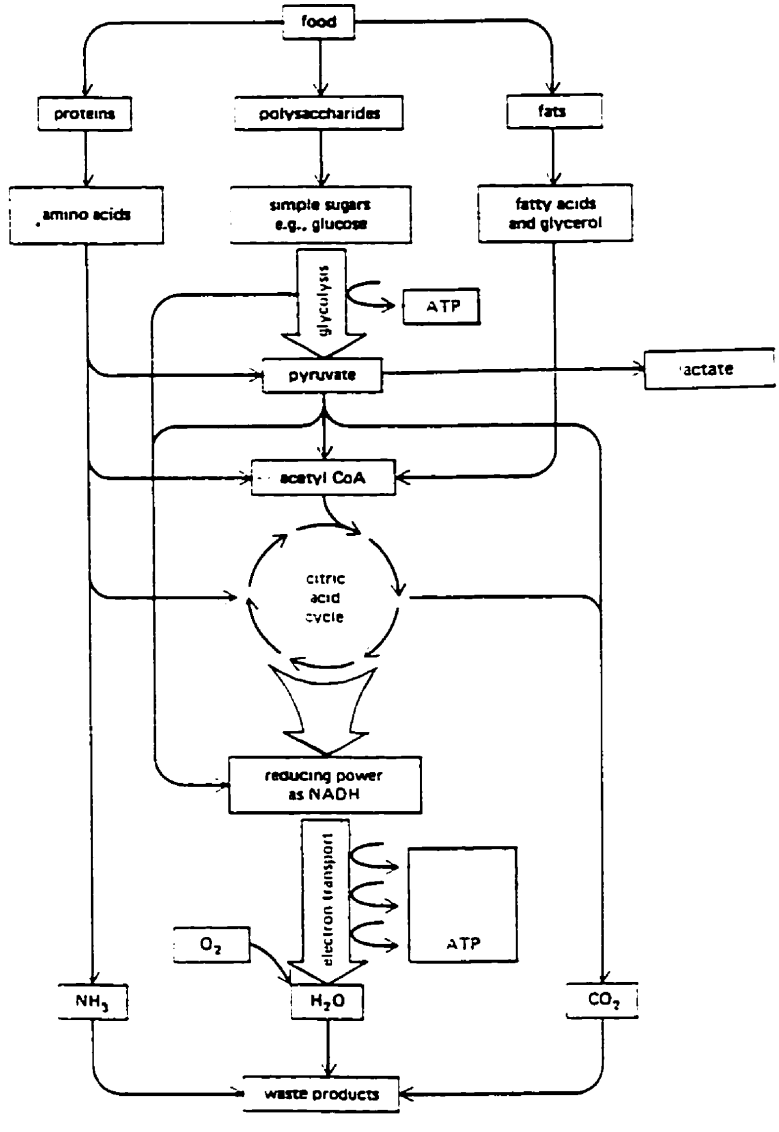

Fig.1.1: Modified schematic of the metabolic conversion of proteins, polysaccharides, and fats via aerobic respiration (glycolysis and citric acid cycle) and anaerobic respiration pathways (conversion to lactate). ("Molecular Biology of the Cell", edited by Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. New York: Garland Publishing Inc, 1983.)

Alternative evidence has suggested that lactic acid accumulation is not the only cause of tumor acidity. Newell *et al.* (1993) and Yamagata and Tannock (personal communication) found that two types of variant glycolysis deficient cells, implanted in mice, grew tumors that were as acidic as those derived by parental cells that produced high levels of lactate. Only two types of acid are probably produced in sufficient quantity to lower pHe: lactic acid and carbonic acid. Carbonic acid is a product of the hydration of CO_2, which dissociates to bicarbonate and H^+. (Hochachka and Mommsen, 1983) Gullino *et al.* (1965) observed increased levels of both CO_2 and lactate in measurements of tumor interstitial fluid in rats. In addition, Martin and Jain (1994) observed that hypercapnia (10% CO_2) alone, like hyperglycemia (6g/kg) alone, resulted in a 0.3 pH unit decrease in intersititial pH in VX2 carcinoma tumors.

1.4 Methods for Measuring pHe and pHi In Tumors

1.4.1 Extracellular pH

A) pH Sensitive Microelectrodes

The efficacy of using glass electrodes to measure pH has been known since the beginning of the century. Measurements of tissue environmental pH are made by determining the voltage difference generated across the pH (H^+) sensitive glass membrane, placed in the sample, and the pH-insensitive reference electrode, placed in buffer of known pH. (Bates, 1981) Microelectrodes

have been manufactured with tip diameters ranging from 3 μm to 5mm. (Carlsson and Acker, 1988) These electrodes can be inserted directly into tissue, and measurements are thought to be primarily of pHe. However, the invasive insertion of the electrode may result in tissue damage - consequently contributing to the pHe measured. (Wike-Hooley *et al.*, 1984) It is possible to measure the pH of different regions of the tumor (the heterogeneity) by advancing the electrode through the tumor tissue in small increments. (Jahde and Rajewsky, 1982, Carlsson and Acker 1988) However, the microelectrodes that are used in tumors have a sensitive area ranging from 100 to 500μm; which makes it difficult to detect differences in pHe within microregions of tumors, since the distance between necrotic regions and blood vessels is smaller than the size of the electrode.

B) Micropore Chambers

Gullino *et al.* (1965) measured the interstitial pH of tumors in rats by utilizing spherical porous chambers. The chambers, approximately 1cm in diameter and possessing pore sizes of 0.1-0.5 μm, were coated with cells and then implanted into subcutaneous tissue. As the tumor tissue grew around the embedded chamber, interstitial fluid accumulated within the compartment, which could be withdrawn with a syringe and analyzed. One disadvantage of this method is that it is not possible to measure the heterogeneity of pH within the population of cells in a tumor. In addition, since measurements cannot be made unless the chamber acquires sufficient fluid (50-200μL) - kinetic pH measurements are not possible.

C) Fiber Optics

pH measurements can also be made by using the pH-sensitive dye phenol red. (Van de Merwe *et al.*, 1990) The indicator is contained in a chamber at the tip of a 0.7mm diameter fiber optic probe sheathed in a nylon catheter. pH measurements are based on the ratio of the absorbance at pH-independent (>600nm) to pH-dependent (558nm) wavelengths. The probe responds linearly within the range of pH 6.3-7.8. Although the electrode is limited to this measurement range, this is not a major limitation since pHe's outside this range are not found commonly in solid tumors. (Wike-Hooley *et al.*, 1984) Like glass electrodes, consequent disruption of tissue from insertion could affect direct pH measurements. Furthermore, the large diameter of the tip makes it difficult to measure the heterogeneity of pH within tumors.

1.4.2 Intracellular pH

A) ^{31}P Magnetic Resonance Spectroscopy

Inorganic phosphate, which is predominantly intracellular, exists in the following equilibrium:

$$H^+ + HPO_4^{2-} \Longleftrightarrow H_2PO4^- \quad (pKa\ 6.9)$$

Since this equilibrium is pH-dependent, it is possible to determine pHi by monitoring the ionization status of inorganic phosphate (Pi). (Moon and Richards, 1973) Inorganic phosphate

gives a unique peak in the NMR spectrum, and the position, or chemical shift σ, is dependent on the proportion of HPO_4^{2-} to $H_2PO_4^-$ in a sample - which, in turn, is dependent on pH. (Ng *et al.*, 1982) The relative σ of the Pi peak to a control, such as phosphocreatine, may be used to determine pHi. (Fig 1.2) A calibration curve is constructed by measuring the σ difference of solutions of known Pi and phosphocreatine (containing physiological concentrations of ions. (Ng *et al.*, 1982) Subsequently, the pHi of a solution may be extrapolated from such a curve. With this method it is possible to estimate pHi in tumor volumes as small as $100mm^3$ with a resolution of ~0.05pH units. (Koutcher *et al.*, 1987)

NMR spectroscopy is advantageous in that it is non-invasive and allows for repeated measurements as a function of time. The major drawback is that the method has poor spatial resolution - which prevents the measurement of pHi among subpopulations of cells within a tumor. It is also difficult to obtain absolute tumor spectra due to contamination from surrounding (normal) tissue signals.

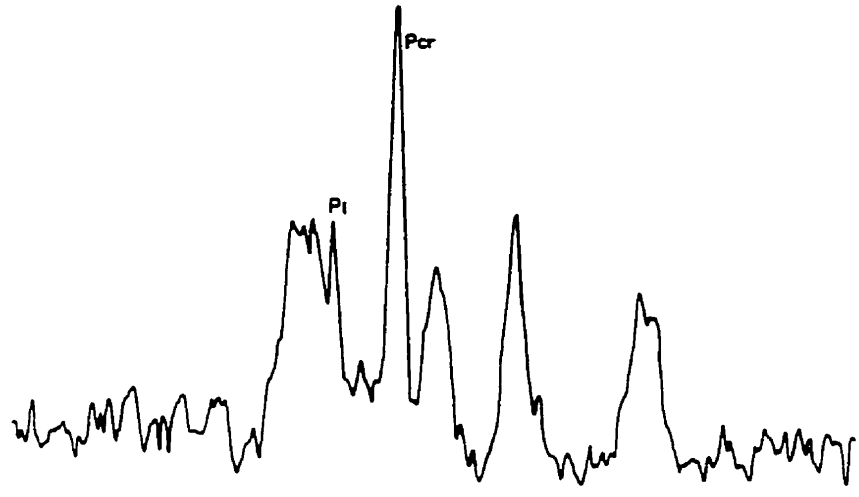

Fig. 1.2: *In Vivo* ^{31}P NMR spectroscopy of MOPC 104E myeloma cells. The intensity of the peaks is proportional to the relative number of the corresponding phosphorous atoms. (Ng *et al.*, 1982)

B) Positron Emission Tomography

This method, using ^{11}C-5,5-dimethyl-2,4-oxazolidinedione (DMO), is an adaptation of the long used method of estimating pHi of cells *in vitro* using the equilibration of weak acids or bases (see section 1.5 C) and allows for the measurement of pHi in tissue. (Rottenberg *et al.*, 1984) Since ^{11}C-DMO is a weak acid (pKa 6.1), if it is assumed that the extra and intracellular concentrations of the uncharged forms of the weak acid will equilibrate, then tissue pH can be calculated using the Henderson-Hasselbalch equation. pH is obtained by measuring the ^{11}C-DMO concentration and estimating the fractional volume of extracellular water of the tissue. Thus far this technique has been used only to measure pHi in normal and malignant brain tissue; it has not been adopted widely due to the difficulties of estimating water content precisely, and poor spatial resolution (~2cm^3).

C) Flow Cytometry

Hedley and Jorgensen (1989) developed a method in which rodent tumors were disaggregated into a buffer, which did not contain Na^+ and HCO_3^-, in the presence of amiloride, to prevent changes in pHi. The pHi of the cells was measured in a flow cytometer after they were stained with BCECF (see section 1.5 D). Mean pHi and the variation in pHi of tumor samples were

determined from fluorescence comparisons with calibration curves using nigericin (see section 1.5 D).

One limitation of this technique is the difficulty in preventing changes in pHi between the time of tumor excision, disaggregation, and pHi measurement (~30minutes) - which may lead to artefacts in measurement.

1.5 Methods of Measuring pHi *In Vitro*

A) Microelectrodes

pH-sensitive microelectrodes (tip diameter ~1μm), which can be inserted into individual cells, have been developed. (Roos and Boron, 1981) This method has the potential to monitor continuously the pH in single cells. However, the invasive nature of the electrode could cause destruction of the cell (or components of the cell) resulting in inaccurate pHi measurements.

B) ^{31}P NMR Spectroscopy

The method described previously for the measurement of pHi of tumor tissue can be used to evaluate the pHi of cells in suspension, or in spheroids. (Freyer *et al.*, 1990, Freyer *et al.*, 1991)

The technique is advantageous because it is non-invasive, and allows the monitoring of other phosphate containing metabolites, such as ATP and phosphocreatine. However, large cell numbers are required for adequate measurement, which may lead to problems with nutrient supply and catabolite removal; for spheroids, these problems may be overcome with the use of perfusion chambers. (Freyer *et al.*, 1990)

C) Distribution of Weak Acids or Bases

This method is based on the assumption that only the uncharged forms of weak acids (or bases) permeate the membrane of cells, and that these undissociated forms will equilibrate across the plasmallema. The pHi can then be determined from knowledge of the pHe, pKa, and the concentration of acid [HA] both inside and outside the cell. Weak acids exist in the following equilibrium: $HA \Longleftrightarrow H^+ + A^-$. From the Henderson- Hasselbalch equation we can determine:

$$pHe = pKa + \log \frac{[A^-]e}{[HA]e}$$

$$pHi = pKa + \log \frac{[A^-]i}{[HA]i}$$

When the uncharged forms of the weak acid equilibrate: [HA]e = [HA]i, and hence the above equations can be combined to calculate pHi.

From the above equations we obtain the following:

$$[HA]i = \frac{[A^-]i}{10^{pHi-pKa}} \quad \text{and} \quad [HA]e = \frac{[A^-]e}{10^{pHe-pKa}}$$

Combining the above expressions we obtain the following:

$$[A^-]i = [A^-]e(10^{pHi-pHe})$$

Therefore: $$pHi = pKa + \log \frac{[A^-]e(10^{pHi-pHe})}{[HA]e}$$

Most weak acid studies have been conducted with 5,5-dimethyl-[2-^{14}C]-oxazolidine-2,4-dione (DMO, pKa 6.1). Experimentally, [HA]e and [HA]i are estimated by measuring ^{14}C-DMO radioactivity associated with cells, and that associated with supernatant in a cell suspension. (Waddell and Butler, 1959) However, since the method assumes that [HA]e and [HA]i are equal, it may only be necessary to measure one value.

This technique is simple and can be applied to a variety of cells. However, since cells are damaged during the measurement procedure, it is impossible to perform repeated estimations of pHi. In addition, kinetic studies of rapid changes in pHi cannot be performed because of the time needed for equilibration. Moreover, weak acids and bases diffuse into organelles, which gives a pH reading that is a composite value for several cell compartments, and not the pHi of the tumor cytosol. (Rink *et al.*, 1982)

D) pH-sensitive Fluorescent Dyes

Fluorescein derivatives are popular pH-sensitive dyes for monitoring internal pH. (Boron, 1989) The most commonly used derivative is 2',7'-bis-(2-carboxyethyl) 5-(and 6)-carboxyfluorescein (BCECF) which is obtained in the uncharged, non fluorescent acetoxymethyl ester form (BCECF-AM). BCECF-AM can diffuse easily into the cell where non-specific esterases in the cytosol cleave off the ester groups - leaving the charged and highly fluorescent molecule BCECF. (Fig.1.3) (Musgrove *et al.*, 1986, Molecular Probes, 1996)

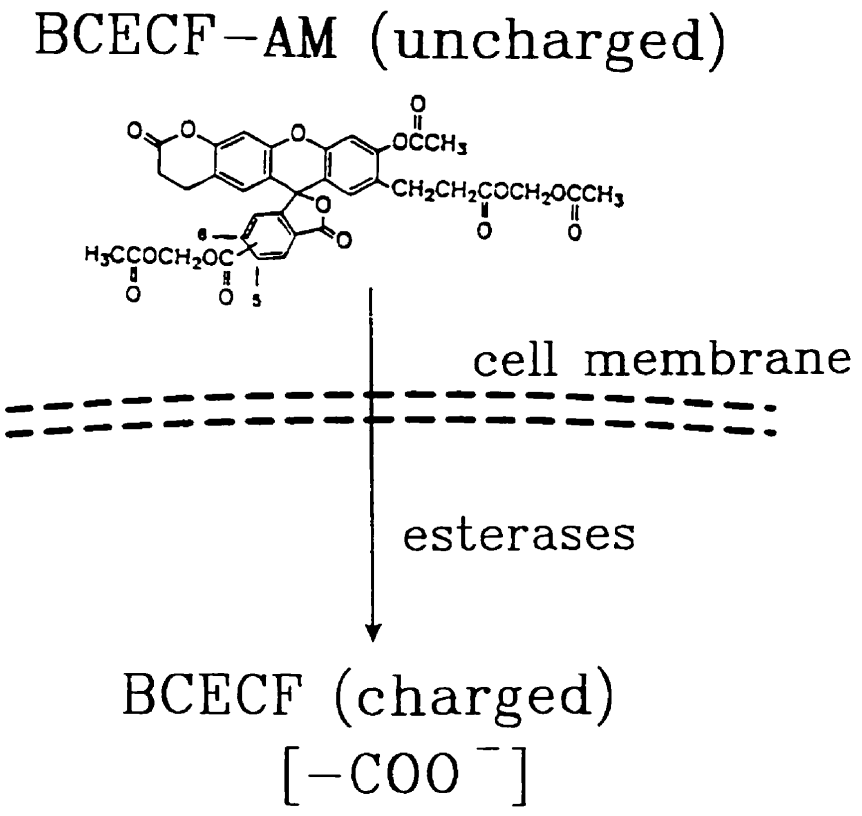

Fig.1.3: Diagram of cleavage of the acetoxymethyl ester group of BCECF-AM in the cell cytoplasm.

The pKa of BCECF (6.9) is useful for monitoring pH in the physiological range. BCECF also has the advantage that it is non-toxic at its optimum concentration of 2μg/ml for up to two hours of exposure. BCECF, and other derivatives of fluorescein have also been found to distribute evenly within the cytoplasm and nucleus, without entering organelles - eliminating the influence of organellar pH. (Thomas *et al.*, 1979, Musgrove and Hedley, 1990)

Following excitation at 488nm, the intensity of BCECF fluorescence is linearly dependent on pHi in the range of 6.0 to 7.6. (Musgrove *et al.*, 1986, Rotin *et al.*, 1987) This behavior is true for both BCECF emission and excitation fluorescence, which makes it versatile for both fluorometry and flow cytometry.

i) Fluorometry

The pHi may be determined by observing the ratio of fluorescence measured at pH-dependent to pH-independent wavelengths. The use of a ratio is advantageous because it can overcome the effects of such factors as dye leakage, cell size, or structure. (Musgrove *et al.*, 1986, Boyer and Hedley, 1994) In fluorometry, a ratio of excitation intensities is easily measured. A ratio of BCECF excitation at 495nm (pH-dependent) to 440nm (pH-independent), at emission 525nm gives a measure of pHi. (Fig.1.4)

ii) Flow Cytometry

Ratiometric pHi measurements are also conducted in flow cytometry. Unlike fluorometry, flow cytometry assesses emission wavelength intensities through the use of single excitation lasers (e.g. the 488nm argon laser) Using BCECF, pHi is measured from a ratio of the fluorescence emission at 525nm (pH-dependent) to 640nm (pH-independent), at excitation 488nm. (Fig.1.4)

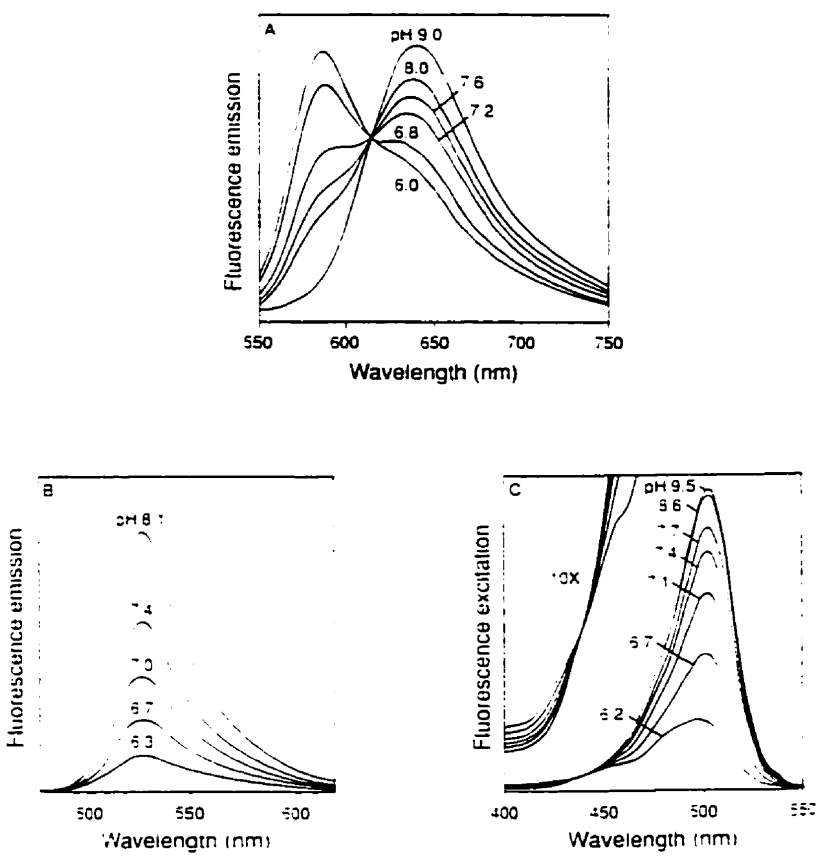

Fig.1.4: A. Fluorescence emission spectra of SNARF-1 at various levels of pHi at excitation ~488nm. B. Fluorescence emission spectra of BCECF at various levels of pHi at excitation ~488nm. C. Fluorescence excitation spectra of BCECF at various levels of pHi at emission ~525nm. (Molecular Probes, 1996)

Other fluorescent dyes, such as 2,3-dicyanohydroquinone (DCH; pK 8.0) (Musgrove *et al.*, 1986), and semicarboxyseminapthorhodafluor (SNARF-1; pK 7.4) (Van Erp *et al.*, 1991, Chow *et al.*, 1996) have also been used to measure pHi. DCH is not as versatile as BCECF because it requires the use of UV excitation, which is not routinely used, and it leaks rapidly from cells (50% within 30 minutes). It's pK is also not ideal for measuring pHi in the physiological range. (Van Erp *et al.*, 1991, Boyer and Hedley, 1994) SNARF-1 sensitivity (~0.003pH units) and resolution (~0.3pH units) are similar to BCECF. Unlike BCECF, SNARF-1 undergoes a pH-dependent shift in wavelength from orange-yellow at acidic pH, to red at basic pH. This allows for the assessment of pH using ratiometric measurements of the dye at two emission wavelengths: 580nm and 640nm. (Fig.1.4) (Van Erp *et al.*, 1991, Molecular Probes, 1996) However, SNARF-1 has generally been found to possess better resolution at more alkaline pH (~7.0-8.0) than BCECF, which has been used at a more acidic range (~6.0-7.6).

iii) Calibration Curves for Fluorometry and Flow Cytometry

As with ^{31}P spectroscopy, the pHi of a sample is extrapolated from a calibration curve. The reference curve is derived usually from cells in high K^+ ($[K^+]e$) solutions buffered to known pHe, using the ionophore nigericin. Nigericin is a K^+/H^+ ionophore that shuttles these ions across the membrane to equilibrate the ratio of:

$$\frac{[K^+]e}{[K^+]i} = \frac{[H^+]e}{[H^+]i}$$

If it is assumed that $[K^+]i = [K^+]e$, then $[H^+]i = [H^+]e$, and the intracellular pH will equal extracellular pH. The change in fluorescence ratio is plotted against pHi (equal to the pHe), and the fluorescence of a sample can be extrapolated from the calibration curve - to obtain pHi. This method may be used in both the fluorometer, and the flow cytometer. (Boyer and Hedley , 1994)

For calibration, cells are exposed to buffers with $[K^+]e$ concentrations of approximately 140mM - which is the assumed intracellular concentration of K^+ in most cells. (Thomas *et al.*, 1979) This is probably a valid assumption for most cell types since the pHi values obtained are similar to those determined by other methods. (Thomas *et al.*, 1979, Rink *et al.*, 1982, Wang *et al.*,1990)

iv) Influence of K^+ Concentration on Calibration

In order to determine the influence of $[K^+]e$ on calibration curves using BCECF, I undertook experiments to compare those curves generated for mouse mammary carcinoma (EMT6), Chinese hamster ovary (CHO), and murine fibrosarcoma (KHT) cell lines using fluorometry. (Refer to Chapter Two, section 2.4.1, for results) These studies demonstrate that the experimental use of 140mM K^+ solutions is not unreasonable because the method does not appear to be very sensitive to the values of $[K^+]i$ that is assumed. Although not apparent in the cell lines above, the slope of the calibration curve has been found to vary between samples, cell types, and dose and time responses to nigericin. Hence, the nigericin calibration method is not useful when knowledge of absolute pHi values are crucial. Nevertheless, the nigericin calibration method is sufficient for

determining relative values of and changes in pHi, which will be pursued in the studies outlined in this thesis.

In general, the advantages to utilizing fluorescent probes are: the ability to detect rapid changes in pHi with minimal damage to cells, the high sensitivity which allows for the detection of small changes in pHi, and low toxicity at their optimum concentrations.

E) Pseudo-Null Calibration

pHi can be extrapolated by deriving a calibration curve using a method proposed by Chow *et al.* (1996) for use with flow cytometry. It is based on the null point method originally described by Eisner *et al.* (1989). The method uses cells loaded with BCECF or SNARF-1 in mixtures of known concentrations of the weak acid butyric acid (pKa 4.1) and the weak base trimethylamine (pKb 7.8). Only the uncharged forms of the weak acid and base are assumed to permeate the cell membrane, where they donate or accept protons as they dissociate in the cytosol. When cells are exposed to this mixture, the steady state pHi is displaced according to the Henderson-Hasselbalch equation - which translates into a fluorescence signal.

According to Eisner *et al.* (1989), pHi can be derived by determining the mixture of weak acid and base that produces no change in fluorescence pH signal. By exposing cells to various solutions ranging in ratios of acid to base, the ratio that produces no change in pHi is extrapolated

between those that generate the smallest increase/decrease in pHi. The value of pHi at this "null point" is :

$$pHi = pHe - 0.5 \log \frac{[A_T]}{[B_T]}$$

where $[A_T]$ and $[B_T]$ are the total weak acid and base concentrations of the suspension.

However, Chow *et al.* (1996) suggest that the true "null point" is not necessary. When cells are exposed to any ratio of acid to base, the steady-state pHi is displaced and the fluorescence ratio obtained is a measure of this shifted pHi. Given that the molar concentration ratio of acid to base is sufficient, further addition of the same ratio of acid to base causes no further change in pHi. Therefore the displaced pHi is the new null value, which was termed the "pseudo-null" value, which satisfies the above equation.

Unlike the nigericin method where the pHe is varied, the pHe of the buffer used in the null calibration method (Hepes buffer) remains constant at 7.4. Since the pHe is constant, the required ratio of acid to base that must be added to the Hepes buffer to produce the desired pHi can be calculated from the Henderson-Hasselbalch equation.

The advantage of this method is that it is reproducible, and is useful when the knowledge of absolute pHi values is essential. One drawback is that the shift in fluorescence is not stable, and drifts after prolonged exposure (>~3min).

1.6 Regulation of pHi

Measurements of pHe and pHi indicate that although the external pH of most tumors is acidic, the internal pH is close to physiological values. Since cells can survive only if their cytosolic pH is maintained within a narrow range (Wike-Hooley *et al.*, 1984, Musgrove and Hedley, 1990), tumor cells are likely to depend on mechanisms to regulate their internal pH for survival, whereas these mechanisms are less important for normal cells possessing neutral environments.

There are four known mechanisms that regulate pHi in most types of mammalian cells: the buffering capacity of cytosolic and organellar contents, the Na^+/H^+ antiport, the Na^+-independent Cl^-/HCO_3^- exchanger, and the Na^+-dependent Cl^-/HCO_3^- exchanger.

A) Buffering Capacity

Buffering capacity is a measure of the cell's ability to buffer/resist changes in pHi following the addition (or removal) of H^+. This H^+ buffering is considered the immediate cellular pH response to acute acid/base perturbations. (Saleh *et al.*, 1991) There are three known types of intracellular buffering processes: physiochemical, biochemical, and organellar. (Boron, 1989) Physiochemical buffering is achieved by weak acids or bases (usually moities of proteins) within the cell, and the equilibration of neutral forms that can permeate the plasma membrane. Biochemical buffering is a

result of biochemical reactions in the cell that consume or produce H^+ in response to acid or alkaline loads. This is achieved by compounds such as lactate, pyruvate, or citrate. Furthermore, some organelles in the cytoplasm respond to acute acid or alkaline loads by actively pumping H^+ into the cytosol, and decreasing H^+ transport into acidic vesicles (when pHi increases), or the reverse when pHi decreases. (Boron, 1989)

The buffering capacity (β) of a cell is defined as $\beta = \Delta A/\Delta pH$, where ΔA is the amount of acid (or base) that must be added or removed in order to change pHi by ΔpH. (Roos and Boron, 1981, Boron, 1989) The total buffering capacity is comprised of bicarbonate dependent and intrinsic non-bicarbonate components. (Boron, 1989) The bicarbonate buffering capacity is due to the CO_2/HCO_3^- pair, which may contribute up to 60% of the intracellular buffering capacity. The non-bicarbonate capacity is due mainly to the intracellular $H_2PO_4^-/HPO_4^{2-}$ pair, and to titratable groups of amino acids, such as NH_3/NH_4^+, in cellular proteins. (Boron, 1989)

Intrinsic buffering capacity can be determined experimentally by applying a known acid load to cells and measuring the resultant change in pHi. This is commonly achieved using NH_4^+/NH_3. NH_3 equilibrates rapidly across the cell membrane, and with the knowledge of the extracellular concentration of NH_4^+, the pKa, pHi, and pHe, the magnitude of the intracellular acidification can be calculated.

From the above definition of buffering capacity and the Henderson-Hasselbalch equation, it can be

shown (Boyer and Tannock, 1992) that:

$$\beta_i = \frac{[NH_4^+]i}{pHi}$$

where $[NH_4^+]i = \frac{[NH_4Cl]e \cdot (10^{pHe-pHi})}{(1 + 10^{pHe-pK})}$ $\qquad pK = 9.3$ for NH_4^+

Values of intrinsic buffering capacity have been found to range from 10 to 100mM (H^+)/pH unit. (Roos and Boron, 1981)

The bicarbonate buffering component is calculated from the equation: $\beta_{HCO3} = 2.3 \cdot [HCO_3^-]i$. (Boron, 1989) This expression is derived from the Henderson-Hasselbalch equation:

$$pH = pK + \log \frac{[HCO_3^-]i}{sPCO_2}$$

where "s" is the solubility of CO_2 and PCO_2 is the partial pressure of CO_2 in the atmosphere.

From the above equation the following is obtained:

$$[HCO_3^-]i = sPCO_2 \cdot 10^{pHi-pK}$$

Therefore: $$\beta = \frac{d[sPCO_2 \cdot 10^{pHi-pK}]}{d(pHi)}$$

Since PCO_2 is fixed in an open system, PCO_2 can be removed as a constant.

$$\text{Therefore:} \quad \beta = sPCO_2 \cdot \frac{d[10^{pHi-pK}]}{d(pHi)}$$

$$\text{And thus:} \quad \beta = sPCO_2 (\ln 10) 10^{pHi-pK} = 2.3\ [HCO_3^-]i$$

CO_2/H_2CO_3 equilibrates across the cell membrane and the $[HCO_3^-]i$ can be determined from the pHi, pHe, and the $[HCO_3^-]e$ which are known. From the Henderson-Hasselbalch equation, and the knowledge of the other factors, $[HCO_3^-]i = [HCO_3^-]e \cdot (10^{pHe-pHi})$.

Typical β_{HCO3} values have been found to be ~30mM H^+/pH unit under physiological conditions. (Roos and Boron, 1981) The buffering capacity of cells has been found to be substantial when compared to the steady state H^+ concentration (in sub-micromolar range). Nevertheless, buffering is insufficient to providing long-term pHi maintenance because it does not extrude acid (H^+) from the cell.

B) The Na^+-independent Cl^-/HCO_3^- Exchanger

The Na^+-independent Cl^-/HCO_3^- exchanger (Fig.1.5) regulates pHi under alkaline conditions. (Grinstein *et al.*, 1989) Under physiological conditions there is an inward gradient of Cl^-, resulting in the co-transport of HCO_3^- out of the cell thereby leading to cytosolic acidification. The exchanger has been studied extensively in red blood cells, and it has been identified as the

95Kda polypeptide "band 3". (Alper, 1991) It is inhibited by stilbene derivatives such as 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS). The exchanger becomes inactive under acidic cytosolic conditions and therefore, will not be further discussed because of its limited relevance in regulation of pHi in the acidic range. The two major mechanisms which regulate pHi under acidic conditions are the Na^+/H^+ antiport and the Na^+-dependent Cl^-/HCO_3^- exchanger.

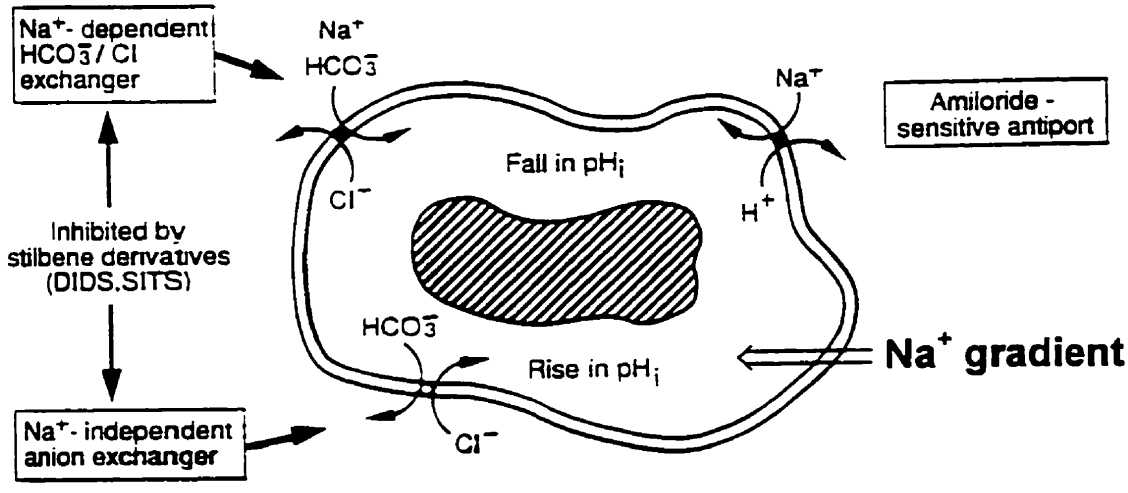

Fig.1.5: Diagram of known membrane-based pHi regulating mechanisms.

C) The Na^+/H^+ Antiport

The Na^+/H^+ transporter (Fig.1.5) is usually quiescent at or around physiological pH, and becomes activated when the cytosol is acidified. (Grinstein *et al.*, 1989) The Na^+/H^+ antiport exchanges

extracellular Na^+ for intracellular H^+ under conditions of low pHi in a 1:1 stoichiometric ratio. (Roos and Boron, 1981) The exchange process is driven by the inward gradient of Na^+; indirectly fueled by the Na^+/K^+ pump, which maintains the Na^+ gradient. (Grinstein *et al.*, 1989) Under artificial conditions where there is an outward gradient for Na^+, such as in the absence of extracellular Na^+, the exchanger operates in reverse. In addition to Na^+ and H^+ the antiport also transports Li^+; a characteristic which has been utilized to derive variants which lack the exchanger. (Pouyssegur *et al.*, 1984, Rotin *et al.*, 1989) The Na^+/H^+ antiport is ubiquitous in mammalian cells. (Sardet *et al.*, 1989) It is inhibited by amiloride and its analogues such as 5-(N-ethyl-N-isopropyl)amiloride (EIPA). (Vigne *et al.*, 1983)

It is known now that there is a gene family of Na^+/H^+ exchanger isoforms termed NHE1-4. These genes show considerable homology in the transmembrane segment of the molecule. (Orlowski *et al.*, 1992) The isoforms differ in their plasma membrane location, molecular weight, and sensitivity to inhibitors (amiloride and its analogs). (Haggerty *et al.*, 1988, Orlowski *et al.*, 1992) The NHE-1 molecule is ubiquitous, and the NHE-2 gene product has been found to be widely distributed in tissues of the gastrointestinal tract, kidney, heart, testes, uterus, and adrenal glands. (Gishan *et al.*, 1995) The other isoforms NHE-3 and 4 seem to be expressed specifically in the gastrointestinal tract. (Orlowski *et al.*, 1992)

The cDNAs encoding the human Na^+/H^+ exchangers NHE-1 and NHE-2 have been cloned and sequenced. The cDNA of the gene encoding NHE-1 has been mapped to human chromosome 1p35-p36.1, while NHE-2 has been located on human chromosome 2q11.1. (Sardet *et al.*, 1989, Szpirer *et al.*, 1994, Gishan *et al.*, 1995) The Na^+/H^+ antiport molecule consists of two distinct

regions: an N-terminal region (~500a.a.) containing 10 transmembrane spanning segments, and a C-terminal hydrophilic cytoplasmic domain. (Sardet *et al.*, 1989) The antiport appears to have a solitary binding site for extracellular Na^+ and a second cytoplasmic "modifier site" that binds to H^+ and controls the exchanger's activity allosterically. (Grinstein *et al.*, 1989) The existence of the allosteric control site allows for the pH modulation of the Na^+/H^+ exchange activity. For example, the modifier site inhibits the antiport when the pHi rises above a certain level - protecting the cell from excess alkalinization. (Grinstein *et al.*, 1989)

Although there is no clear evidence of a direct role of Na^+/H^+ antiport activity and subsequent pHi alkalinization in cell proliferation, the following observations have suggested a connection. (Grinstein *et al.*, 1989) 1. proliferation in invertebrate cells can be promoted by mitogen-free cytoplasmic alkalinization; 2. growth promoting agents such as EGF and TGF-α cause an acute activation of the Na^+/H^+ antiport (in the absence of HCO_3^-) leading to cytosolic alkalinization; (Grinstein *et al.*, 1989, Strazzabosco *et al.*, 1995) 3. amiloride and analogs that inhibit the exchanger have prevented growth factor induced proliferation in some mammalian cells; 4. inhibition of DNA synthesis has been observed in the absence of extracellular Na^+; 5. proliferation in variants deficient in Na^+/H^+ exchange activity is impaired at low pHe, in the absence of HCO_3^-.

In contrast, the following studies challenge the association: 1. the above effects are not observed in the presence of bicarbonate; 2. Na^+/H^+ transporter stimulation is not always mitogenic; 3. cytoplasmic alkalinization does not result in cell proliferation in most cell lines; 4. at physiological

pHe the absence of Na^+/H^+ exchange activity does not hinder mitogenesis, provided that cells are in medium at a permissive pHe. (Grinstein *et al.*, 1989)

The above studies suggest that the Na^+/H^+ antiport and cell alkalinization are not required to induce cell proliferation. Nevertheless, the antiport may be essential when HCO_3^- levels are low, or when the Na^+-dependent Cl^-/HCO_3^- exchanger is not functioning.

Na^+/H^+ exchange activity has been examined under various microenvironmental conditions. Boyer *et al.* (1993) observed an increase in the activity of the Na^+/H^+ antiporter at the center of spheroids derived from murine and human tumor cells, versus the periphery - particularly when exposed to low pHe. Saleh and Batlle (1990) observed that the activity of the Na^+/H^+ antiporter in spontaneously hypertensive rat lymphocytes (SHR) increased with decreasing internal pH. In addition, it was found that proionged exposure (24-48 hours) of renal proximal tubule cells to acidic conditions caused an increase in Na^+/H^+ antiport activity. (Horie *et al.*, 1990) Boyer *et al.* (1993) also observed increased Na^+/H^+ exchanger activity in murine mammary sarcoma (EMT6) and human bladder cancer (MGH-U1) cells exposed to pHe 6.6 for 18 hours.

The importance of the Na^+/H^+ antiport for tumor cell survival was examined by comparing cells of similar genetic background but with different levels of Na^+/H^+ exchange activity. Using the proton suicide technique developed by Pouyssegur's group, Rotin *et al.* (1989) selected variant MGH-U1 human bladder cancer cells which lacked the Na^+/H^+ exchanger. This technique is based on the fact that the Na^+/H^+ exchanger is selective for Li^+ and Na^+. Cells are initially loaded

with Li^+ and then placed in Li^+ free medium at a very low pHe. Since the Na^+/H^+ exchanger is reversible, it will extrude Li^+ along its electrochemical gradient and bring H^+ into the cell. As a result, cells with Na^+/H^+ exchange function should become acidic and die from acid overload, leaving the Na^+/H^+ deficient or inefficient cells. Cells lacking Na^+/H^+ antiport function are presumed to survive because they do not acidify their cytoplasm.

Variant MGH-U1 cells lacking Na^+/H^+ exchange activity produced few tumors in nude mice, which grew only after long latency periods and were found to be comprised of revertant cells. (Rotin *et al.*,1989) Furthermore, Rotin *et al.* found that only revertants regained the ability to generate tumors; which suggests that Na^+/H^+ exchange activity is necessary for survival as solid tumors develop an acidic microenvironment. The results confirm the importance of this antiport in regulating pHi of cells in solid tumors. Nevertheless, further testing of the above hypothesis has not been possible because of difficulties in deriving variant cells with little or no Na^+/H^+ antiport activity from a tumor population, which is often tetraploid.

D) The Na^+-dependent Cl^-/HCO_3^- Exchanger

The Na^+-dependent Cl^-/HCO_3^- exchanger (Fig.1.5) is the other known mechanism that regulates cellular pHi under acidic conditions. (Cassel *et al.*, 1988) The exchanger has been detected in a variety of mammalian cells. (Grinstein *et al.*, 1989) The exchanger transports HCO_3^- into the cell

in exchange for Cl^-, which is extruded against its concentration gradient, probably because the inward gradient of Na^+ allows the transport of $NaHCO_3$ in exchange for HCl. The stoichiometry of the exchanger has been proposed as a 1:1 relationship but the precise mechanism of exchange has not been established. (Roos and Boron, 1981, Grinstein *et al.*, 1989)

The exchanger is very active in tumor cells in the pHe range of 6.5-7.2. (Boyer and Tannock 1992) Like the Na^+/H^+ antiport, the Na^+-dependent Cl^-/HCO_3^- exchanger is inactive at alkaline pHi and becomes activated at acidic pHi. (Grinstein *et al.*, 1989) It was found that at neutral and acidic pHe, the resting pHi of cells was higher in the presence of bicarbonate, than in its absence. (Poole *et al.*, 1964, Cassel *et al.*, 1985) Furthermore, studies reported in this thesis show that in certain cell lines pHi was maintained at a higher level when the Na^+-dependent Cl^-/HCO_3^- exchanger was active as compared to when the Na^+/H^+ antiport was functioning alone. (See Chapter Two, section 2.4.2)

Like the Na^+-independent Cl^-/HCO_3^- exchanger the Na^+-dependent Cl^-/HCO_3^- exchanger is also inhibited by stilbene derivatives such as DIDS. Acidification of cultured cells with ionophores such as nigericin or CCCP leads to toxicity at pHe less than or equal to 6.5, and this effect is enhanced when the Na^+-dependent Cl^-/HCO_3^- exchanger is blocked by DIDS. (Rotin *et al.*, 1987, Newell and Tannock, 1989)

Boyer *et al.* (1992) observed a more pronounced activity of the Na^+-dependent Cl^-/HCO_3^- exchanger than the Na^+/H^+ antiport at pHi values close to physiological, which corroborates

results from other laboratories. (Tonnessen *et al.*, 1990) Yamagata and Tannock (1996) observed that *in vitro* cell killing was more pronounced when tumor cells were exposed to the combination of nigericin and DIDS for 72 hours at pHe 6.8, than when exposed to nigericin and EIPA. The above studies suggest that Na^+-dependent Cl^-/HCO_3^- exchange may play a more dominant role in pHi regulation at levels close to normal resting pHi. (Tonnessen *et al.*, 1990, Boyer and Tannock, 1992, Yamagata and Tannock, 1996)

The Na^+-dependent Cl^-/HCO_3^- exchanger has not yet been characterized at the molecular or genetic level. In addition, there is little knowledge of the importance of the exchanger for survival and growth in tumors. Since both Na^+-independent and dependent Cl^-/HCO_3^- exchange activity are inhibited by the same stilbene derivatives, the Na^+-dependent Cl^-/HCO_3^- exchanger has been compared with the already characterized Na^+-independent Cl^-/HCO_3^- exchanger. However, experimental evidence has shown differential sensitivity of the Na^+-independent and dependent Cl^-/HCO_3^- exchangers to specific inhibitors. Compounds such as: (-)-5c (a [(tetrahydrofluorene-7-yl)oxy]acetic acid derivative) and ethacrynic acid have been found to inhibit selectively Na^+-independent Cl^-/HCO_3^- exchange activity. (Madshus and Olsnes, 1987, Cassel *et al.*, 1988) In addition, it was found that in PS120 cells (lacking Na^+/H^+ antiport function) the Na^+-dependent Cl^-/HCO_3^- exchanger exhibited a 10-fold lower affinity for Cl^- than the Na^+-independent Cl^-/HCO_3^- transporter.

Such analyses have led to the belief that the two exchangers are separate, independent entities, although further studies are needed for confirmation. (Cassel *et al.*, 1988, Grinstein *et al.*, 1989)

In this chapter the measurement of pHe and pHi, the causes of tumor acidity, and mechanisms of pHi regulation have been addressed. The following chapters, in paper format, will explore: the effect of pHe on the level of pHi and on heterogeneity in pHi; determine whether differences in levels of pHi may be exploited for derivation of variant cells; and examine the influence of cell cycle distribution on the pHi distribution in populations of tumor cells. As mentioned previously, low pHe has been found to be a plausible target for tumor-selective therapy. Ergo, it is necessary to understand how within acidic microenvironments tumor cells can maintain their pHi. To achieve this it is imperative to understand the relationship between pHe and pHi, and the variation in pHi within a population of tumor cells. Specifically, since the Na^+/H^+ and Na^+-dependent Cl^-/HCO_3^- exchangers are known to regulate pHi under acidic conditions, it will be useful to determine how exchanger activity affects these relationships. In particular, the study of intercellular variance in pHi may be useful for isolating subpopulations of cells that can regulate their pHi at higher and lower levels; which will give more insight into how such regulation is achieved, and allow for molecular and genetic characterization.

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Chapter 2

Heterogeneity of pHi within Populations of EMT6, CHO, and MCF-7 Cells

2.1 Abstract

Heterogeneity in pHi of populations of murine mammary sarcoma (EMT6), human breast cancer (MCF-7), and Chinese hamster ovary (CHO) cell lines was observed using flow cytometry and the pH-sensitive fluorescent probe BCECF. Cells at various pHe were exposed to: Na^+ or Na^+ and HCO_3^- buffer in the presence or absence of EIPA (an inhibitor of Na^+/H^+ exchange), at pHe 7.4, 7.0, 6.6, and 6.2. This allowed comparison of the relationship between mean pHi, the distribution in pHi values, and pHe within these cell populations when one, both, or neither of the Na^+/H^+ antiport and/or the Na^+-dependent Cl^-/HCO_3^- exchangers were active. pHi was maintained at a higher level when the Na^+-dependent Cl^-/HCO_3^- exchanger was active (~0.1-0.2 pH units higher), with or without Na^+/H^+ exchange activity. Within the pHi range of 6.8-7.3, the activity of the Na^+/H^+ antiport did not influence the level of pHi. However, an effect of the Na^+/H^+ antiport on pHi was observed when cytoplasmic acidification was enhanced in the presence of nigericin - which suggests that its activity becomes important below a pHi threshold. For all three cell lines the coefficients of variation (CV) in pHi (which are an indication of intercellular heterogeneity) were greater than those obtained when cells were exposed to nigericin and high K^+ buffer, indicating that this variability is a true biological effect. Moreover, there was less cell to cell variation in pHi when the Na^+-dependent Cl^-/HCO_3^- exchanger was operating. Under these experimental conditions the Na^+-dependent Cl^-/HCO_3^- exchanger is more active in pHi regulation than the Na^+/H^+ antiport.

2.2 Introduction

Tumors have been known to be heterogeneous with respect to almost all of their phenotypic properties including: cell morphology, karyotype, growth rate, and sensitivity to therapeutic agents. (Dexter and Calabresi, 1982, Heppner and Miller, 1983). Experimental evidence of tumor heterogeneity is abundant; subpopulations have been isolated from every histological type, organ site, and experimental and human tumors. Potential causes of heterogeneity include: variable expression of genes, increased mutation frequency, and microenvironment (as a result of extra-tumor influences). (Heppner, 1984)

Many tumor cells exist within an acidic microenvironment. This is due probably to such factors as the accumulation and the poor removal of metabolic acids (CO_2 and lactic acid). (Tannock, 1968, Sutherland, 1986, Newell and Tannock, 1989, Vaupel *et al.*, 1989) Cells are known to regulate pHi within this acidic milieu via two membrane-based ion exchange mechanisms: the Na^+/H^+ antiport and the Na^+-dependent Cl^-/HCO_3^- exchanger. (Grinstein *et al.*, 1989) The characteristics of both exchangers have been described in the previous chapter, but one of the main driving forces for the activity of the two exchangers is pHi. Both exchangers are minimally active at physiological pHi; the Na^+-dependent Cl^-/HCO_3^- exchanger has been reported to be most active at acidic pHi close to physiological (~6.9) and the Na^+/H^+ antiport at more acidic pHi (~6.6). (Cassel *et al.*, 1988, Grinstein *et al.*, 1989, Boyer and Tannock, 1992)

Variability in extracellular pH in tumors and spheroids has been demonstrated. Martin and Jain (1994) observed decreasing pHe with increasing distance from blood vessels within populations of VX2 carcinomas, using transparent chambers implanted into the ears of rabbits. The chamber design confined the tissue to a thickness of approximately 50μm - allowing for studies of the microcirculation with a microscope to image the chamber with good resolution. (Martin and Jain, 1994) pHe measurements were made using a pulse injection of 0.1mg/mL BCECF into the artery feeding the chamber of the ear. They found that with increasing distance from a blood vessel, the interstitial pH of tumors decreased (0.13pH units over 50μm). Carlsson and Acker (1988) measured pH gradients at different depths in spheroids from seven different types of tumor cells using microelectrodes (tip diameter of about 2-3μm). pHe was measured in 20-25μm increments via stepwise insertion of the microelectrodes to depths of about 200μm within spheroids. They observed a decrease in pHe with increasing distance into spheroids, with a difference of ~0.4 pH units between the periphery and a depth of ~200μm.

Previous studies of pHi as function of pHe have compared average values of pHi within a population. (Fellenz and Gerweck, 1988, Newell and Tannock, 1989, Chu *et al.*, 1990, Boyer *et al.*, 1993) While average values are useful to determine the effects of experimental conditions (vs. steady-state) on the population of cells as a whole, they do not give information on how these conditions affect individual cells within a population. Heterogeneity or variance in tumor pHi makes possible the ascription of pH on a per cell basis, and the association of the magnitude of such values to physiological function or characteristic (i.e. to exchanger activity). (Gillies *et al.*, 1987)

Studies of the variation in pHi at different depths of a spheroid and within tumors at various distances from a blood vessel source have been attempted. (Boyer *et al.*, 1993) The above studies were performed by exposing cells to the DNA stain Hoechst 33342 and the pH-sensitive fluorescent probe BCECF directly in buffer (spheroids) or by lateral tail vein injection (tumors). Using this technique, Boyer *et al.* (1993) were able to show that exchanger activity increased towards the center of EMT6 and MGH-U1 spheroids, especially when grown at pHe 6.6. However, they were not able to assess the variation in pHi of tumors or spheroids relative to their distance from a blood vessel source or from the periphery, respectively, probably due to inadequate staining by Hoechst 33342.

The use of pH sensitive fluorescent probes and flow cytometry makes possible rapid, single cell measurements of pHi. Using flow cytometry and the pH sensitive fluorescent dye BCECF, I have studied the distribution of pHi as a function of pHe within populations of three cell lines: murine mammary sarcoma (EMT6), human breast cancer (MCF-7), and Chinese hamster ovary (CHO) under physiological and acidic conditions; with the purpose of examining the distribution of pHi values in all populations exposed to various pHe conditions.

2.3 Materials and Methods

2.3.1 Cells:

The experiments were conducted with Chinese hamster ovary (CHO), murine mammary sarcoma (EMT6; obtained from Dr. R. Sutherland. Cancer Center for Experimental Therapeutics, Rochester School of Medicine and Dentistry, Rochester, NY), and human breast cancer (MCF-7) cell lines. Cells were maintained routinely in alpha MEM supplemented with 0.1 mg/mL of kanamycin and 10% fetal calf serum. New cultures were re-established from frozen stock every three months. To confirm that cells were free of mycoplasma, routine tests (every 3-6 months) involving direct fluorochrome DNA staining (DAPI) with Hoechst 33258 and assessment with fluorescence microscopy were conducted. (Uphoff *et al.*, 1992)

2.3.2 Reagents:

5-(N-ethyl-N-isopropyl)-Amiloride (EIPA) was synthesized by Research Biochemicals International (Natick, MA). Nigericin, and 2-[N-morpholino]ethane-sulfonic acid (MES) were synthesized by Sigma (St. Louis, MO). Tris(hydroxymethyl)aminomethane (TRIS) was obtained from International Biotechnologies Inc. (New Haven, CN). BCECF-AM, was acquired from Molecular Probes (Eugene, OR). Stock solutions of 1mg/mL BCECF were made with 100% DMSO.

2.3.3 Buffers:

Unless otherwise indicated the following buffers were nominally free of HCO_3^-.

PBS (phosphate buffered saline) was comprised of: 137mM NaCl, 3mM KCl, 2mM KH_2PO_4, 8mM Na_2HPO_4, 1mM $CaCl_2$, and 1mM $MgCl_2$.

K^+ solutions were buffered to the required pH by combining ratios of the following two solutions: one that contained 110mM K_2HPO_4 and 20mM NaCl, and the other that consisted of 135mM KH_2PO_4 and 20mM NaCl.

Na^+ solutions contained: 140mM NaCl, 5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 5mM glucose, and were buffered to the required pH with 20mM MES and 20mM TRIS.

Na^+ and HCO_3^- solutions contained: 25mM $NaHCO_3$, 115mM NaCl, 5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 5mM glucose. All solutions of HCO_3^- were prepared in advance without $NaHCO_3$, which was added before use, and then buffered to the required pH using 0.5N HCl.

2.3.4 Instrument Used

Experiments were conducted using a Coulter Epics Elite Flow Cytometer (Coulter Electronics, Hialeah, FL, USA) equipped with an argon laser to excite BCECF fluorescence at 488nm. Fluorescence emission was measured at 525nm and 640nm, and the electronic ratios of the two signals were assessed.

2.3.5 Assessment of the Relationship Between pHi, and Distribution in pHi, and pHe

Approximately 1.6 X 10^7 cells were centrifuged and resuspended in PBS and stained with 2μg/mL BCECF at 37°C for 30 minutes. (Musgrove *et al.*, 1986, Rotin *et al.*, 1987) Aliquots of

approximately 10^6 cells were transferred to separate test tubes and the cells were then re-centrifuged and suspended in the following solutions: Na^+ solution to select for Na^+/H^+ antiport function, Na^+ buffer in the presence of EIPA (10μM final concentration) to inhibit both exchangers, Na^+ and HCO_3^- buffer to allow both exchangers to operate, and Na^+ and HCO_3^- buffer in the presence of EIPA to select for Na^+-dependent Cl^-/HCO_3^- exchanger activity. Using flow cytometry, the average pHi values and the distribution of pHi values within populations of tumor cells were observed at pHe 7.4, 7.0, 6.6, and 6.2.

The determination of the pHi of a sample population is explained in detail in Chapter One (section 1.5 D). The distribution of (or cell to cell variation in) pHi values within a population of cells was measured by the coefficient of variation or CV. (Hedley and Jorgensen, 1989) The CV is determined by the following equation:

$$CV = \frac{\text{standard deviation of population sampled}}{\text{mean channel number}} \times 100$$

(Coulter, 1994)

CV has no units and is a percentage.

The instrument calculates the above from the following criteria in the population sampled:

$$\text{mean channel number} = \frac{\Sigma\,(\text{channel number} \times \text{cell count in the channel})}{\text{area of the distribution}}$$

where area of the distribution is equivalent to the total cell count of the population sampled.

$$\text{standard deviation} = \sqrt{\frac{\Sigma\,[(\text{channel number} - \text{mean})^2 \times \text{cell count in the channel}]}{\text{area of the distribution}}}$$

(Coulter, 1994)

An increase in CV should reflect an increase in pHi variation. However, the calculations for CV are based on the assumption that the actual pHi distributions obtained from experimental samples are normally distributed around the mean pHi value. The reliability of the CV value is questionable if the actual pHi distributions deviate greatly from normal (ie. are grossly skewed). To alleviate the problem of grossly skewed distributions, an estimate for a 1/2CV value can be used. The instrument calculates this value by the following equation:

$$1/2CV = \frac{42.46 \times \text{width of peak at half the peak height}}{\text{median peak position}}$$

(Coulter, 1994)

where 42.46 is an adjustment factor determined for the instrument.

However, the estimation of 1/2CV is based on the assumption that, regardless of the morphology of the actual sample distribution, the shape of the distribution at 1/2 peak height is symmetrical around the median peak. In my experimental observations the actual pHi distributions have been approximately normally distributed, and therefore CV calculations are sufficient.

The ability to discern the heterogeneity in pHi of a population of tumor cells is dependent on the ability of the machine to distinguish the fluoresence signals reflecting variation in pHi from background noise (i.e. its resolution). The influence of background alone can be determined by placing cells in conditions where the pHi will equal the pHe, i.e. by exposing cells to nigericin and high $[K^+]e$ solution. Nigericin is an ionophore that is known to equilibrate the internal and external concentration ratios of K^+ and H^+, making pHi equal pHe. Since nigericin will clamp the pHi to equal pHe, under ideal conditions - a single pHi distribution peak reflecting pHe is expected. Any breadth in the distrbution is a measure of the level of background noise in the signal. (Fig.2.1) (Hedley and Jorgensen, 1989, Chu *et al.*, 1990)

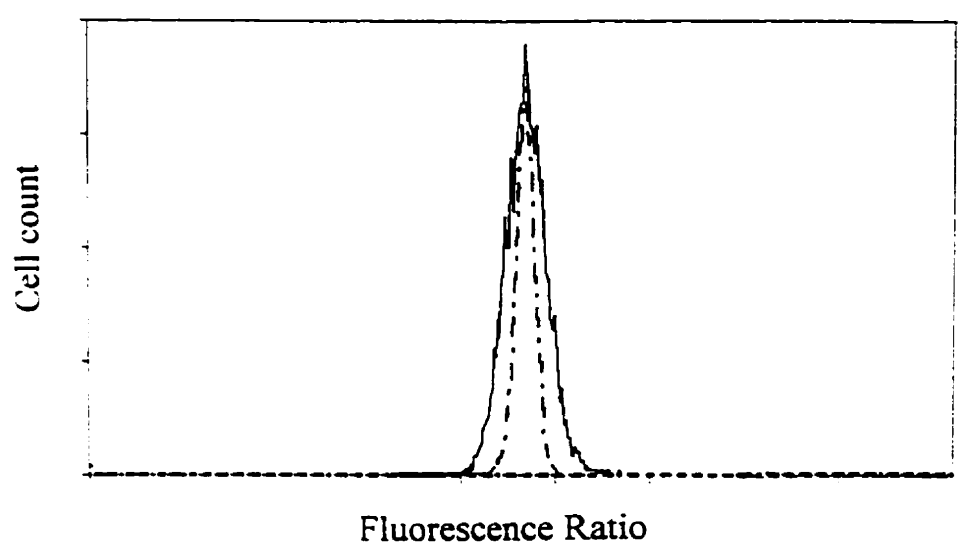

Fig.2.1: Representative fluorescence ratio histograms of cells measured in the absence (solid line) and presence (broken line) of high K^+ buffer (140mM) and nigericin(10μM). The heterogeneity in pHi of a population of cells is determined by comparing the coefficient of variation (CV; a measure of intercellular variation) in pHi in the presence and absence of high K^+ buffer and nigericin. (See section 2.3.5)

Resolution can be estimated by comparing the overlap between the breadth in pHi distributions of samples differing in pHi. (Musgrove and Hedley, 1990, Boyer and Hedley, 1994) Overlay calibration histograms of fluorescence ratio vs pHi distributions for EMT6 cells in high $[K^+]e$ buffer in the presence of nigericin revealed good separation in the means of pHi in the range of 7.4-6.4; i.e. within the experimental range of interest. (Fig.2.2)

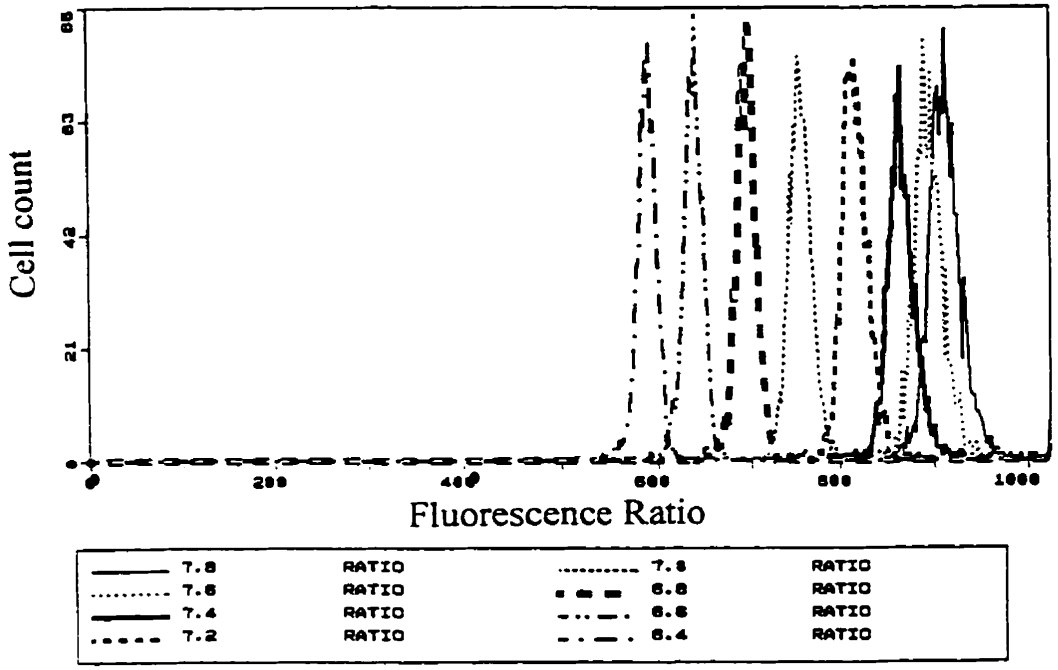

Fig.2.2: Histograms of flow cytometric fluorescence ratio vs pHi for EMT6 cells stained with 2μg/mL BCECF and exposed to high K^+ buffer (140mM) at pHe 7.8-6.4 in the presence of 10μM nigericin. The figure shows good resolution between the peaks for pHe values ≤ 7.4.

2.4 Results

2.4.1 Preliminary Experiments of the Influence of K^+ Concentration on Calibration

Murine mammary sarcoma (EMT6), murine fibrosarcoma (KHT), and Chinese hamster ovary (CHO) cells were exposed to varied $[K^+]e$ concentrations (70mM to 180mM), in the presence of 10μM nigericin. At $[K^+]e$ concentrations around 140mM (130-150mM), it was found that the fluorescence intensity ratios at a given pHi value were similar. At even higher and lower concentrations of $[K^+]e$, it was observed that there was slight variation in fluoresence ratio values. Furthermore, it was found that in EMT6 cells, using 70mM and 180mM $[K^+]e$ generated calibration curves that extrapolated to pHi values that were different than the pHe of the external buffer. For example, the fluorescence ratio (pHi) value of cells exposed to 70mM K^+ buffer at pHe 7.4 corresponded to a fluorescence ratio (pHi) value of ~7.2 in cells exposed to 130-150mM $[K^+]e$ buffered to pHe 7.4. (Fig.2.3)

2.4.2 Preliminary Experiments to Optimize conditions

Preliminary experiments of EMT6 cells stained with 0.2μg/mL, 1μg/mL, and 2μg/mL BCECF in high K^+ buffer (140mM) and nigericin (10μM) showed optimum resolution at the highest concentration used. (Fig. 2.4) For optimum fluorescence conditions, I stained cells with 2μg/mL BCECF (which is equivalent to a final concentration of 4μM) in PBS initially in the absence of

fetal calf serum. For experiments, cells were subsequently centrifuged and exposed to buffers containing dialyzed fetal calf serum to enhance cell viability and to minimize the influence of electrolyte contributions from the fetal calf serum.

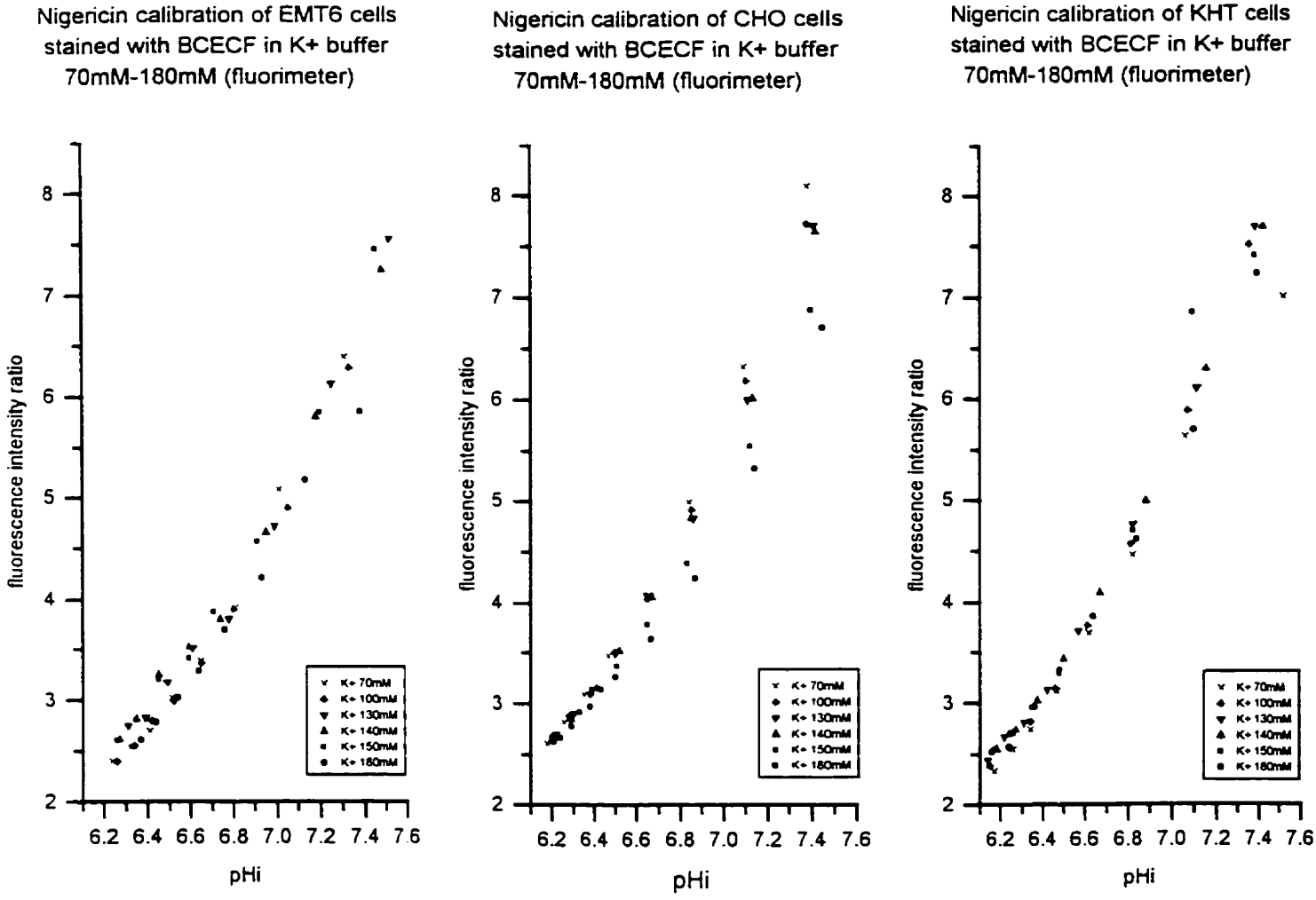

Fig.2.3: EMT6, CHO, and KHT cells stained with 2μg/mL BCECF in K^+ buffer concentrations ranging from 70mM - 180mM in the presence of nigericin(10μM).

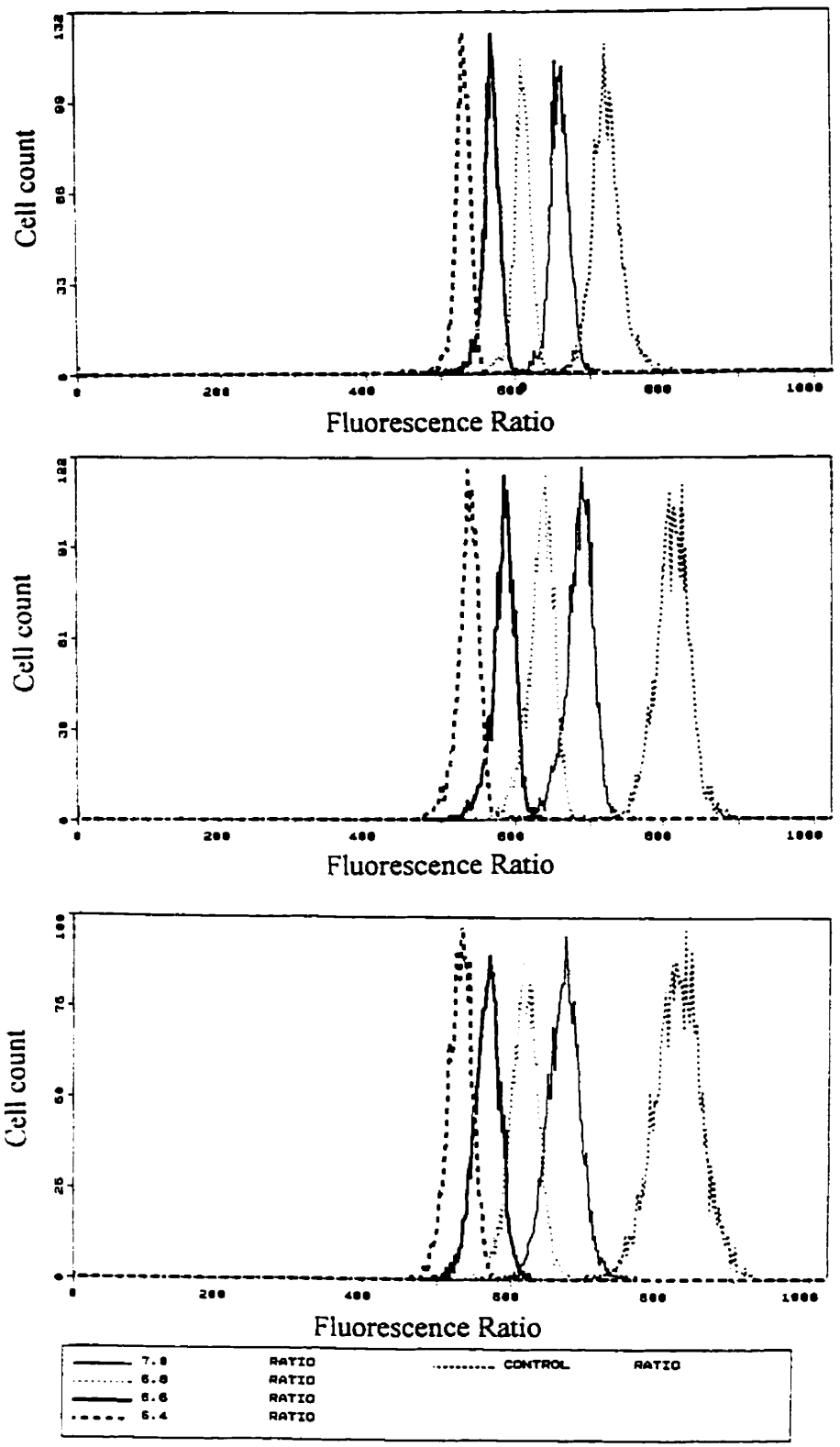

Fig.2.4: Histograms of fluorescence ratio vs pHi for EMT6 cells stained with 0.2μg/mL (bottom panel), 1μg/mL (middle panel), and 2μg/mL (top panel) BCECF and exposed to high [K^+] (140mM) buffer at pHe 7.0-6.4 in the presence of 10μM nigericin. Control cells were exposed to PBS.

2.4.3 Relationship between pHi and pHe

In EMT6 and CHO cell lines pHi was maintained at a relatively higher level under conditions which allowed activity of both exchangers or Na^+-dependent Cl^-/HCO_3^- exchanger activity; (~0.1-0.2 pH units higher) than when neither exchanger or only the Na^+/H^+ antiport were active. (Fig.2.5, 2.6)

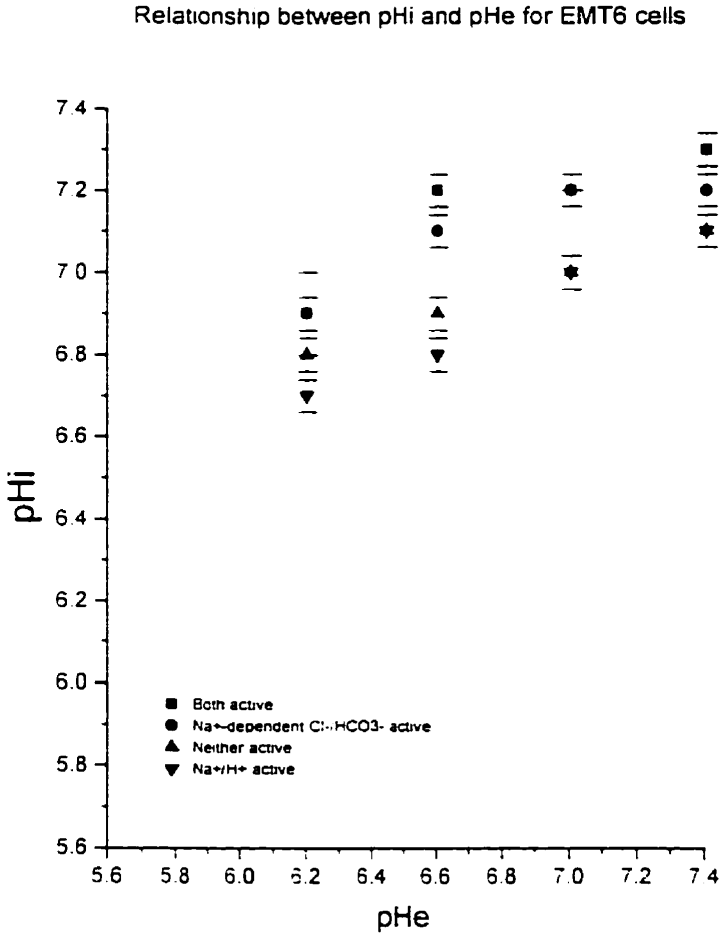

Fig.2.5: Relationship between pHi and pHe of EMT6 cells assessed by BCECF staining and flow cytometry. Cells were placed for 5 minutes in the following conditions: 1) Na^+ and HCO_3^- buffer to select for both Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- exchanger function. 2) Na^+ and HCO_3^- buffer in the presence of 10μM EIPA to select for Na^+-dependent Cl^-/HCO_3^- exchanger activity. 3) Na^+ buffer in the presence of 10μM EIPA to preclude either exchanger operation. 4) Na^+ buffer to select for Na^+/H^+ antiport operation. (Error bars represent S.E.; n=5)

Greater differences in pHi values were observed in EMT6 cells, especially at pHe 6.6, where separation between high and low values was ~0.3 pH units. MCF-7 cells maintained pHi at relatively higher overall levels than EMT6 or CHO cells at low pHe; exposure to pHe of 6.2 reduced pHi to only ~7.1. (Fig.2.7)

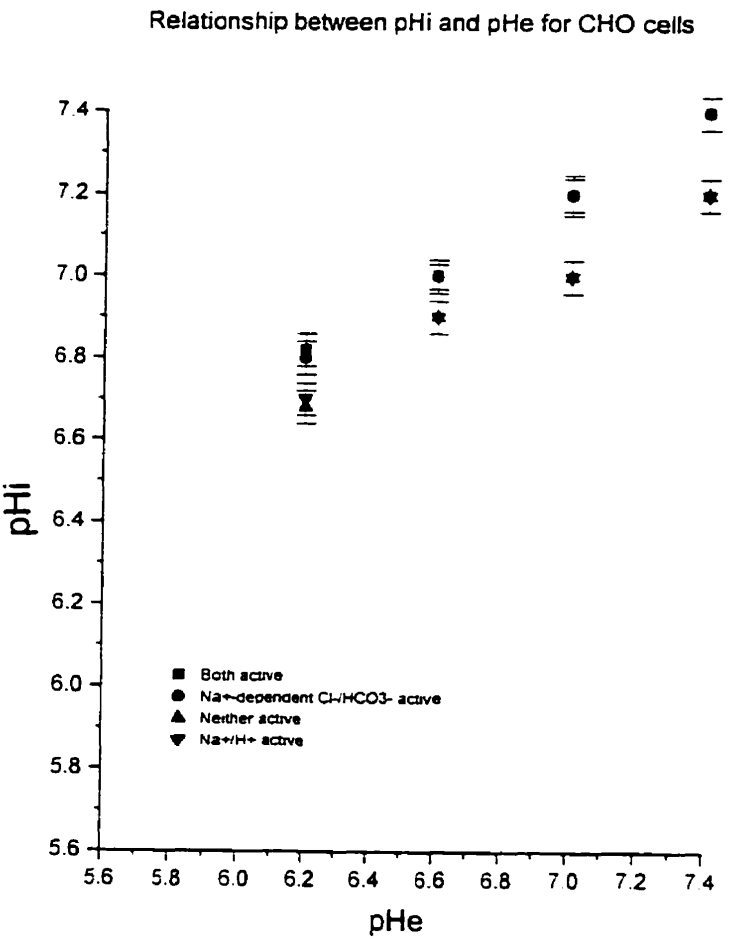

Fig.2.6: Relationship between pHi and pHe of CHO cells assessed by BCECF staining and flow cytometry. Cells were placed for 5 minutes in the following conditions: 1) Na^+ and HCO_3^- buffer to select for both Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- exchanger function. 2) Na^+ and HCO_3^- buffer in the presence of 10μM EIPA to select for Na^+-dependent Cl^-/HCO_3^- exchanger activity. 3) Na^+ buffer in the presence of 10μM EIPA to preclude either exchanger operation. 4) Na^+ buffer to select for Na^+/H^+ antiport operation. (Error bars represent S.E.; n=5)

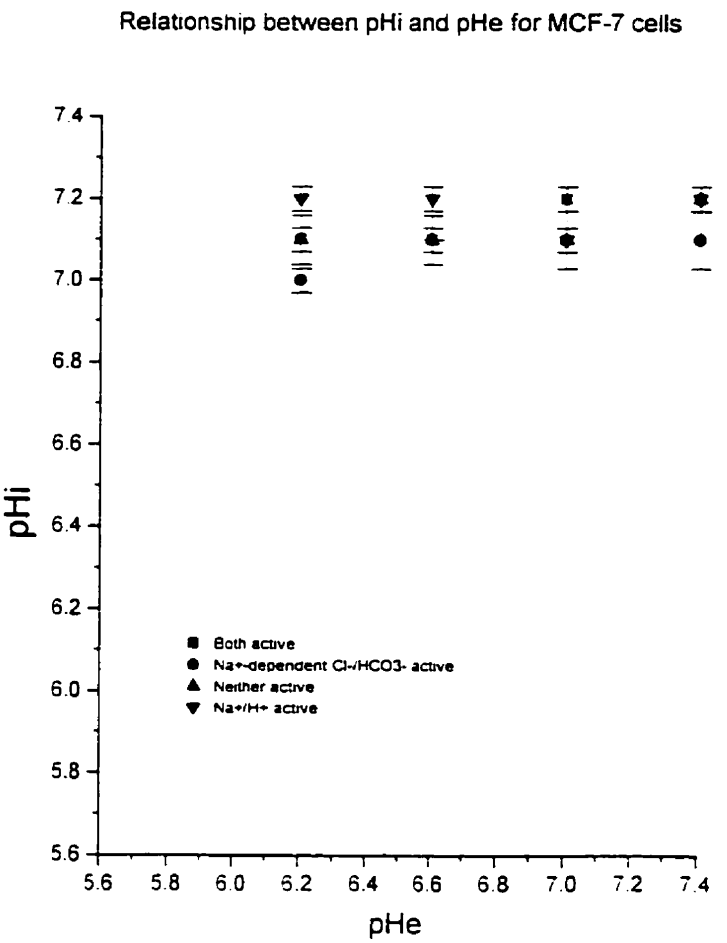

Fig.2.7: Relationship between pHi and pHe of MCF-7 cells assessed by BCECF staining and flow cytometry. Cells were placed for 5 minutes in the following conditions: 1) Na^+ and HCO_3^- buffer to select for both Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- exchanger function. 2) Na^+ and HCO_3^- buffer in the presence of 10μM EIPA to select for Na^+-dependent Cl^-/HCO_3^- exchanger activity. 3) Na^+ buffer in the presence of 10μM EIPA to preclude either exchanger operation. 4) Na^+ buffer to select for Na^+/H^+ antiport operation. (Error bars represent S.E.; n=5)

When cellular pHi acidification was enhanced by exposing cells to the same buffers in the presence of nigericin, pHi was still maintained at a higher level when the Na^+-dependent Cl^-

/HCO_3^- exchanger was active than when the Na^+/H^+ antiport was operating alone. Nigericin acidification increased the differences in level of pHi maintained under the different conditions. (Fig. 2.8, 2.9) It was also found that pHi was maintained at a higher level when the Na^+/H^+ antiport alone was active than when neither exchanger was operating at pHe 7.0 and 6.6 in EMT6 cells.

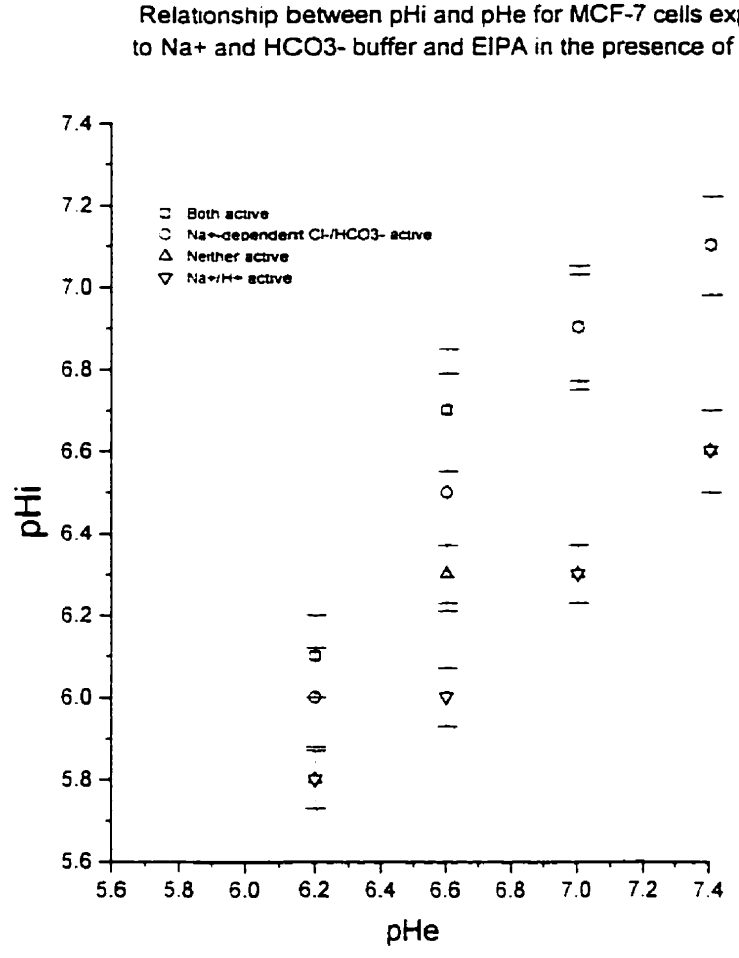

Fig.2.8: Relationship between pHi and pHe of MCF-7 cells assessed by BCECF staining and flow cytometry in the presence of 10μM nigericin. Cells were placed for 5 minutes in the following conditions: 1) Na^+ and HCO_3^- buffer to select for both Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- exchanger function. 2) Na^+ and HCO_3^- buffer in the presence of 10μM EIPA to select for Na^+-dependent Cl^-/HCO_3^- exchanger activity. 3) Na^+ buffer in the presence of 10μM EIPA to preclude either exchanger operation. 4) Na^+ buffer to select for Na^+/H^+ antiport operation. (Error bars represent S.E.; n=5)

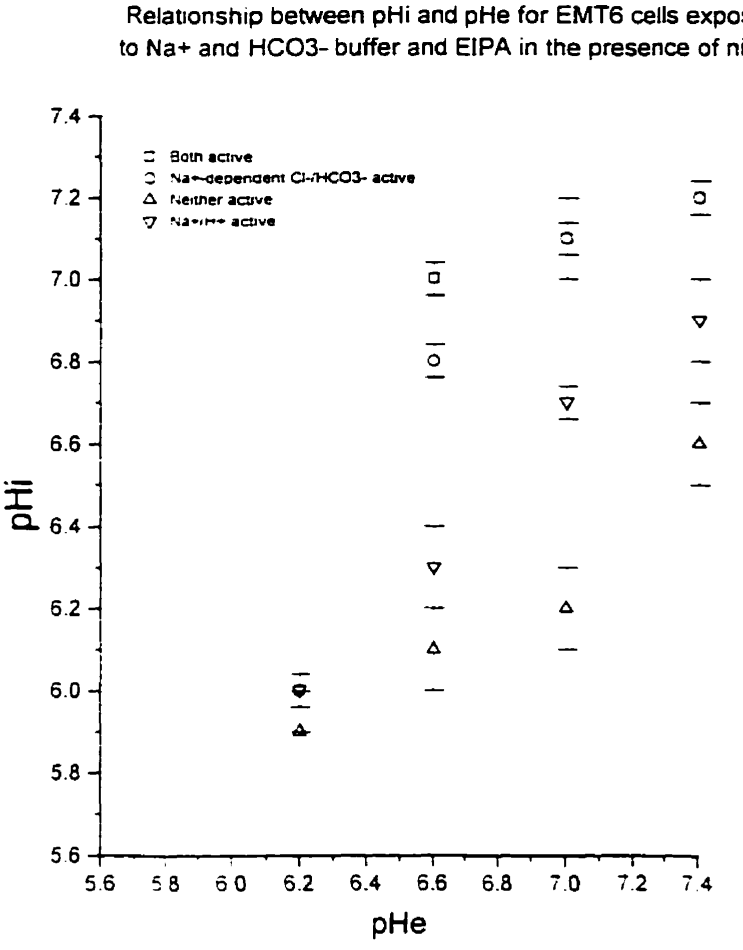

Fig.2.9: Relationship between pHi and pHe of EMT6 cells assessed by BCECF staining and flow cytometry in the presence of 10μM nigericin. Cells were placed for 5 minutes in the following conditions: 1) Na^+ and HCO_3^- buffer to select for both Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- exchanger function. 2) Na^+ and HCO_3^- buffer in the presence of 10μM EIPA to select for Na^+-dependent Cl^-/HCO_3^- exchanger activity. 3) Na^+ buffer in the presence of 10μM EIPA to preclude either exchanger operation. 4) Na^+ buffer to select for Na^+/H^+ antiport operation. (Error bars represent S.E.; n=5)

2.4.4 Variation in pHi vs pHe

The relationship between the variation (CV) in pHi and pHe for the three cell lines is shown in Figs. 2.10 to 2.12. In all three cell lines the coefficient of variation (CV) ranged from approximately 6% - 10%; increasing with decreasing pHe (and as a result - pHi). The highest

values of ~10% were observed in EMT6 cells exposed to pHe 6.2. Overall, MCF-7 cells maintained pHi at a higher level and possessed lower variance (CV) than CHO or EMT6 cells. The greatest separation was observed in EMT6 cell lines exhibiting a difference of ~4% in CV between pHe 7.4 and 6.2 when cells were exposed to conditions to encourage only Na^+/H^+ antiport function. Moreover, the coefficient of variation obtained when the Na^+-dependent Cl^-/HCO_3^- exchanger was active was less than when the Na^+/H^+ antiport was in operation.

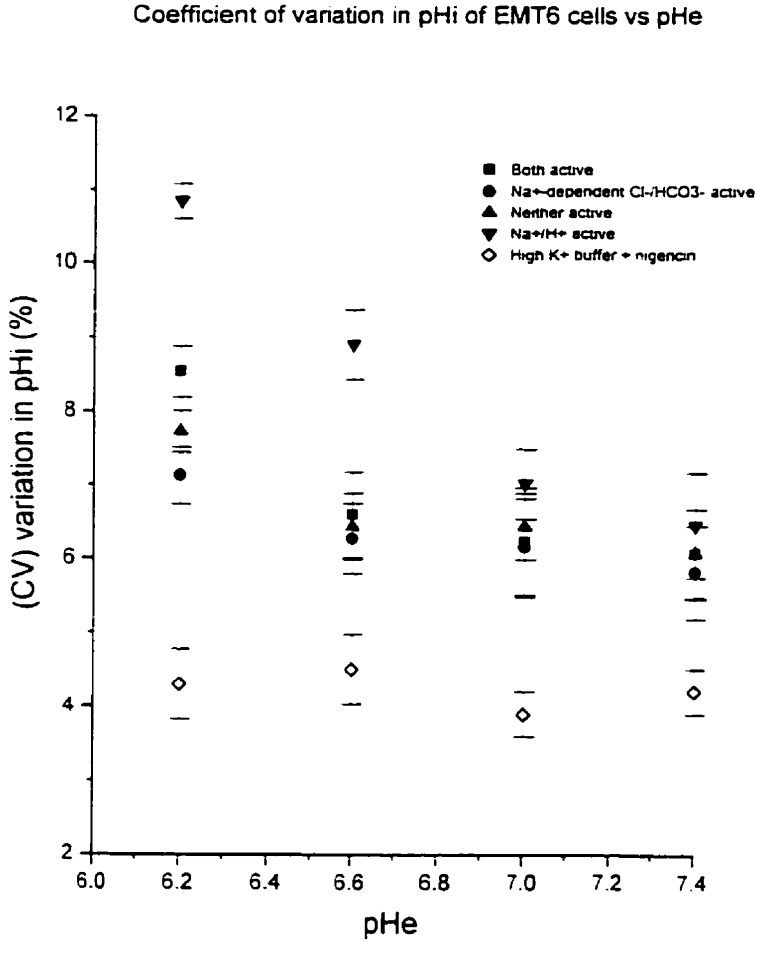

Fig.2.10: Coefficient of variation (for distribution in pHi) vs pHe for EMT6 cells exposed for 5 minutes to the following conditions: 1) Na^+ and HCO_3^- buffer to select for both Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- exchanger function. 2) Na^+ and HCO_3^- buffer in the presence of 10μM EIPA to select for Na^+-dependent Cl^-/HCO_3^- exchanger activity. 3) Na^+ buffer in the presence of 10μM EIPA to preclude either exchanger operation. 4) Na^+ buffer to select for Na^+/H^+ antiport operation. The coefficient of variation vs. pHe when EMT6 cells were exposed to high K^+ buffer (140mM) and nigericin (10μM) has also been included. (Error bars represent S.E.: n=5)

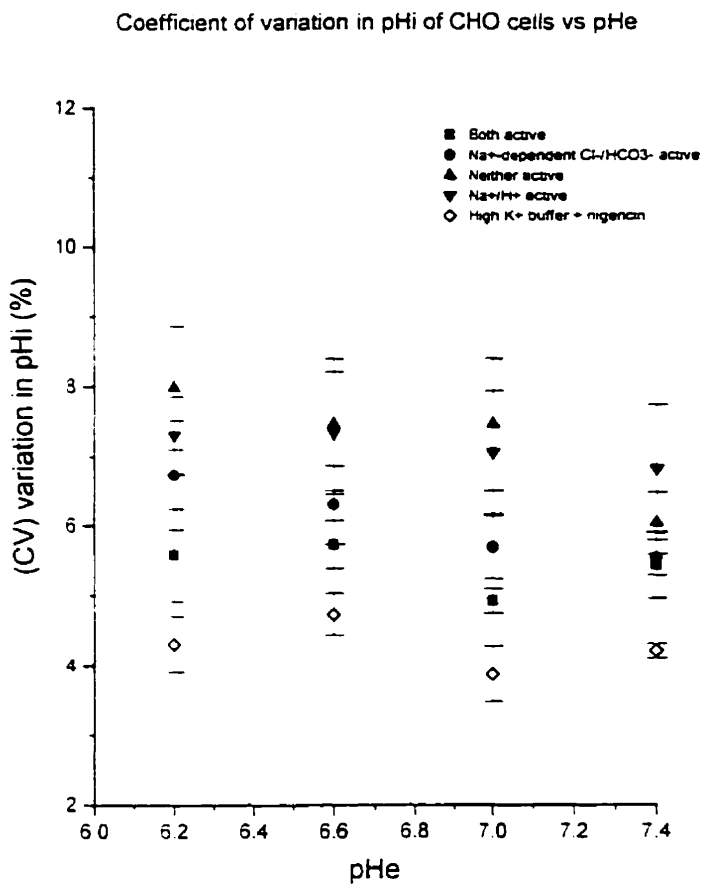

Fig.2.11: Coefficient of variation (for distribution in pHi) vs pHe for CHO cells exposed for 5 minutes to the following conditions: 1) Na^+ and HCO_3^- buffer to select for both Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- exchanger function. 2) Na^+ and HCO_3^- buffer in the presence of 10μM EIPA to select for Na^+-dependent Cl^-/HCO_3^- exchanger activity. 3) Na^+ buffer in the presence of 10μM EIPA to preclude either exchanger operation. 4) Na^+ buffer to select for Na^+/H^+ antiport operation. (Error bars represent S.E.; n=5) The coefficient of variation vs. pHe when CHO cells were exposed to high K^+ buffer (140mM) and nigericin (10μM) has also been included. (Error bars represent S.E.; n=5)

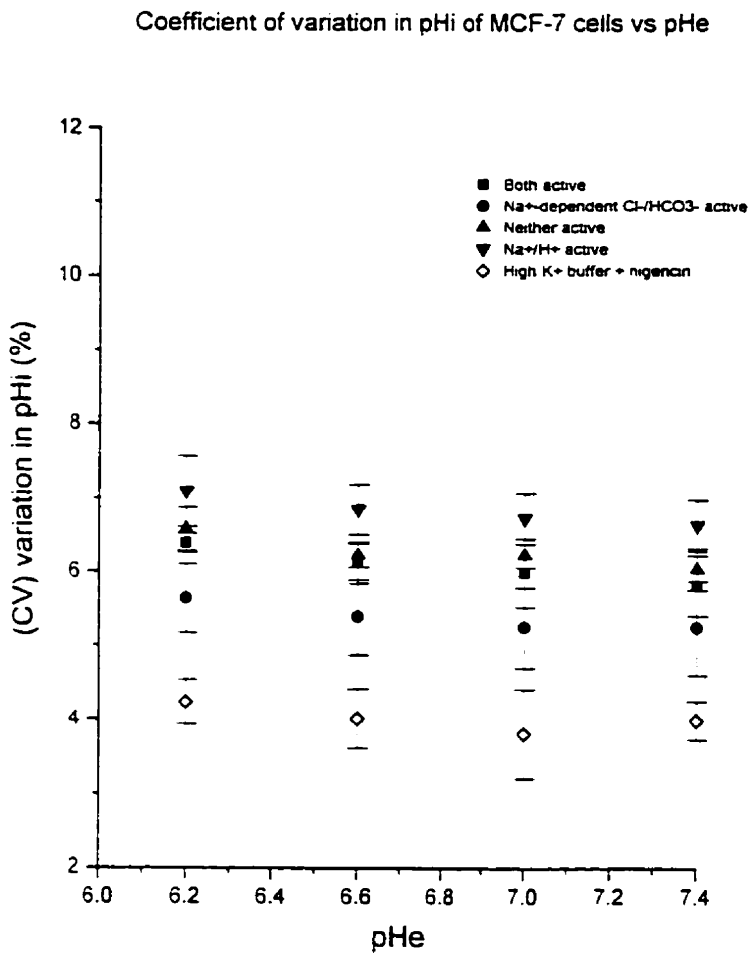

Fig.2.12: Coefficient of variation (for distribution in pHi) vs pHe for MCF-7 cells exposed for 5 minutes to the following conditions: 1) Na^+ and HCO_3^- buffer to select for both Na^+/H^+ antiport and Na^+-dependent Cl^-/HCO_3^- exchanger function. 2) Na^+ and HCO_3^- buffer in the presence of 10μM EIPA to select for Na^+-dependent Cl^-/HCO_3^- exchanger activity. 3) Na^+ buffer in the presence of 10μM EIPA to preclude either exchanger operation. 4) Na^+ buffer to select for Na^+/H^+ antiport operation. (Error bars represent S.E.; n=5) The coefficient of variation vs. pHe when MCF-7 cells were exposed to high K^+ buffer (140mM) and nigericin (10μM) has also been included. (Error bars represent S.E.; n=5)

Consistently, it was found that the distribution in pHi values of the populations sampled was greater than when cells were exposed to high $[K^+]e$ (140mM) buffer in the presence of nigericin. (Fig.2.13) The average CV values were: ~7.0% (EMT6), ~6.5% (CHO), ~6.1% (MCF-7).

These values coincide with variation in pHi of +/- 0.2 pH units within the cell populations. Average CV values in the presence of nigericin (in high $[K^+]e$ buffer) were significantly smaller than those in the absence of nigericin (~4.2%, ~4.3%, ~4.0%, respectively). (Student t-test, $p \leq 0.005$)

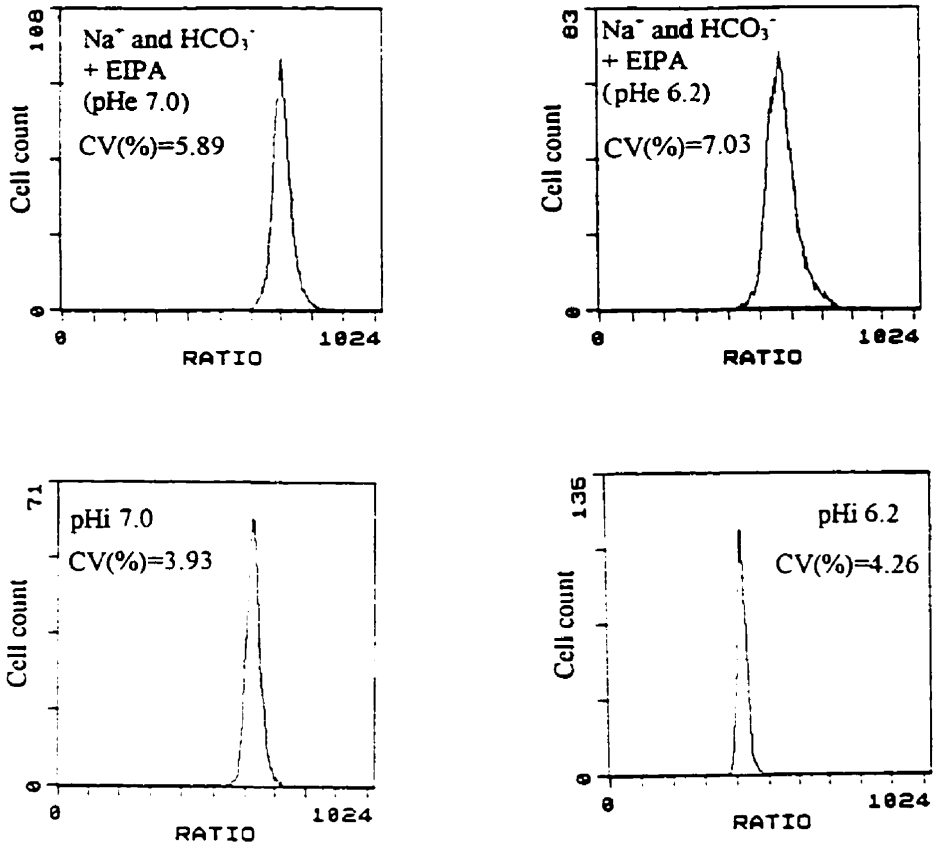

Fig.2.13: Sample pHi distributions of EMT6 cells exposed to either: Na^+ and HCO_3^- buffer and EIPA (10μM) or high K^+ (140mM) buffer and nigericin (10μM), at pHe 7.0 and 6.2. Note the narrower peaks in the presence of nigericin.

The standard deviation (S.D.) of the sample populations (as an alternative measure of variability) was also considered (data not shown). With this approach, the S.D. of the pHi distributions of the test samples (S.D.: EMT6 ~43.7, CHO ~40.1, MCF-7 ~39.9) were ~2X greater than samples exposed to high $[K^+]e$ buffer and nigericin (S.D.: ~23.2, ~23.9, ~22.5, respectively). Although the CVs were observed to increase at low pHe, the corresponding SD values were similar for the various experimental conditions.

2.5 Discussion

The above results suggest that under the experimental conditions used, all three cell lines are relying more on Na^+-dependent Cl^-/HCO_3^- exchange for regulation of pHi. Within the pHi ranges observed (~6.8-7.4), the operation of the Na^+-dependent Cl^-/HCO_3^- transporter is not unexpected. For example, Boyer *et al.* (1992) observed that Na^+-dependent Cl^-/HCO_3^- exchange activity was more dominant in the pHi range of 6.7-7.2. This suggests that within the natural acidic interstitial environment of cells (mean pHe ~6.9) EMT6, CHO, and MCF-7 cells are more likely to depend on the Na^+-dependent Cl^-/HCO_3^- exchanger to regulate pHi. Studies conducted *in vivo* showed greatest tumor cytotoxicity when drugs were used to inhibit the ability of this exchanger to function. (Yamagata and Tannock, 1996)

Within the pHi range observed, the Na^+/H^+ antiport did not seem to influence the level of pHi; which indicates that the antiport might not have been operating in the experimental range of

exposure. This was probably due to the fact that reduction in pHi values under the experimental conditions to a minimum value of ~6.8 (CHO cells) was not sufficient to influence the pHi threshold of the Na^+/H^+ antiport. For example, Boyer *et al.* (1992) observed only minimum operation of the Na^+/H^+ antiport at pHi 6.7-7.2; with greater activity at more acidic levels. Because all three cell lines used are known to possess Na^+/H^+ transporter activity, it is assumed that further decreases in pHi should induce antiport operation.

Biological heterogeneity of a population of cells can be assessed by comparing cells in the presence and absence of high $[K^+]e$ buffer and nigericin. The significantly greater relative CV values of cells exposed to conditions in the absence of nigericin and high $[K^+]e$ buffer suggest that the influence of machine noise is minimal, and that the cell to cell variation observed is a true biological effect. This phenomenon is further substantiated because when cells were exposed to Na^+ or Na^+ and HCO_3^- buffer(with or without EIPA) in the presence of 10μM nigericin a decrease in the distribution of pHi (CV) was observed (data not shown), although not to as great an extent as in high $[K^+]e$ buffer.

The greater heterogeneity in pHi when cells were placed in buffers in the absence of nigericin was also reflected in the significantly higher SD of the sample populations vs. those cells exposed to high $[K^+]e$ buffer and nigericin.

Studies within populations of B16 melanoma tumors , Chinese hamster ovary cells (CHO), and neuroblastomas (NG108-15) have suggested that the populations exhibited little heterogeneity in

pHi. (Hedley and Jorgensen, 1989, Chu *et al.*, 1990) For example, Hedley and Jorgensen (1989) found that the mean coefficient of variation for B16 tumors was ~9.56% which translated to a variance in pHi within the population of +/- 0.4 pH units. However, the variance observed in the presence of nigericin was ~9.20% - almost as large as the test samples; suggesting that the range of pHi values observed was associated with the experimental variations.

Variation in behavior of different cell lines is one explanation of the differences in my results as compared to previous studies. Unlike the observations in tumors and spheroids of Hedley and Jorgensen (1989) and Chu *et al.* (1990), respectively, my experiments were conducted *in vitro*. Moreover, my cell lines were subjected to different sample preparation and experimental conditions. Hedley and Jorgensen (1989) and Chu *et al.* (1990) exposed cells to choline chloride and fresh medium, respectively; while I placed cells in Na^+ or Na^+ and HCO_3^- buffer at various pHe. Furthermore, with the technique used by Hedley and Jorgensen (1989), which applies *in vitro* pH measurement methods *in vivo*, it is difficult to prevent changes in pHi and to maintain heterogeneity in pHi from the time of tumor excision to pHi measurement (see Chapter One, section 1.4.2 C); which could explain their inability to distinguish heterogeneity in pHi from background noise. Also, Chu *et al.* (1990) stained cells with a final BCECF concentration of 1μM; which is suboptimum and might have precluded sufficient staining before exposure to medium containing fetal calf serum, which possesses esterases.

Although an increase in CV was observed with decrease in pHe (pHi), SD values were similar. This discrepancy could be a result of the corresponding change in H^+ concentration ($[H^+]$) with

change in pHi. Since pHi is a negative log scale, a lower pH value is equivalent to a greater H^+ concentration (i.e. a change of one pH unit = 10 fold change in $[H^+]$). Hence a constant S.D. may still result in a greater distribution (CV) in pHi of a sample population at low pH due to the increase in $[H^+]$. It is uncertain whether the increase in CV is a reflection of increase in heterogeneity. The inherent problem with both CV and 1/2CV calculations is that the values are numerically dependent on the denominator (mean/median peak position). This poses problems when experimental situations result in a change in the peak position. A difference in ~200-225 channel numbers (~1 pH unit) could result in a ~1.5X difference in CV values, regardless of the existence of a difference in standard deviations of the populations sampled.

An ideal comparison, to overcome this problem, is to consider the pHi distributions of all experimental conditions that have been adjusted to the same median/mean peak position. This could probably be accomplished by constant adjustment of photomultiplier tube (PMT) voltage outputs for each experimental condition. However, this constant manipulation of the PMTs may introduce additional artefacts.

In conclusion, under my experimental conditions, the Na^+-dependent Cl^-/HCO_3^- exchanger is dominant in pHi regulation. Furthermore, the greater distribution in pHi among samples of EMT6, CHO, and MCF-7 cells is a reflection of biological heterogeneity in pHi. However, it is inconclusive whether the heterogeneity in pHi increases at low pHe (pHi) and further testing is suggested.

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Chapter 3

Can Heterogeneity in pHi be Exploited for Derivation of Variant Cells with Different Levels of Na^+-dependent Cl^-/HCO_3^- Exchanger Activity?

3.1 Abstract

Flow cytometry and the pH-sensitive fluorescent probe BCECF were utilized in attempts to derive variant cells with increased and decreased Na^+-dependent Cl^-/HCO_3^- exchanger activity. Populations of murine mammary sarcoma (EMT6), human breast cancer (MCF-7), and Chinese hamster ovary (CHO) cell lines were sorted on the basis of increased and decreased levels of pHi in Na^+ and HCO_3^- buffer in the presence of EIPA. Subpopulations with highest and lowest values of pHi were collected and propagated in selective media (alpha MEM at pH 7.1 and 7.4) to encourage the continued separation in levels of exchanger function. After several rounds of selection, exchanger activity of control vs sorted populations was compared using recovery from NH_4Cl-acid load. In addition, control and sorted-and-expanded populations of EMT6 cells were analyzed for cell cycle distribution and pHi using SNARF-1 and Hoechst 33342, and cell cycle distribution *per se* with propidium iodide DNA staining. Control cells and cells selected for increased and decreased levels of pHi and expanded in culture appeared to possess similar cell cycle distributions. Furthermore, it was observed that there was no change in pHi as control and selected cells progressed through the cycle. Acid load recovery studies did not reveal any significant difference in level of Na^+-dependent Cl^-/HCO_3^- exchange activity between control and sorted populations for any of the three cell lines. These results suggest that variability in pHi is due to random stochastic variation in the expression of exchanger function, and that this method does not appear to be useful for the selection of variant cells with increased or decreased Na^+-dependent Cl^-/HCO_3^- exchanger activity.

3.2 Introduction

Tumor cells often exist in a more acidic microenvironment than normal cells; this is a result of such factors as the accumulation and insufficient removal of metabolic acids (CO_2 and lactic acid). (Wike-Hooley *et al.*, 1984, Newell and Tannock, 1989, Vaupel *et al.*, 1989) To overcome the more stressful environment there is evidence that tumor cells rely on two exchangers to regulate the pH of their internal milieu under acidic conditions: the Na^+/H^+ antiport and the Na^+-dependent Cl^-/HCO_3^- exchanger. (Grinstein *et al.*, 1989) Both are described in detail in Chapter One, sections 1.6 C and D. Previous work on these exchange mechanisms, predominantly the Na^+/H^+ antiport, have suggested the potential for targeting exchangers for tumor-selective therapy. One extensively studied approach is the use of drugs to selectively block the exchange mechanisms. Previous work has shown that drugs such as: 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) to block the Na^+/H^+ antiport, and 4,4'-diisothiocyanatostilbene 2,2'-disulfonic acid (DIDS) to prevent Na^+-dependent Cl^-/HCO_3^- exchanger operation result in tumor cell kill when used with agents that acidify tumor cells. (Rotin *et al.*, 1987, Luo and Tannock, 1994, Yamagata and Tannock, 1996)

The importance of mechanisms of pHi regulation in determining tumor cell survival (and their potential for tumor-selective therapy) may be assessed by comparing parental cells with variant cells that have increased or loss of exchanger function. This approach has been used by Rotin *et al.* (1989) for human bladder carcinoma cells (MGH-U1). Rotin *et al.* (1989) derived variant MGH-U1 cells which lacked the Na^+/H^+ antiport using the proton suicide technique. (Pouyssegur *et al.*, 1984) When variant MGH-U1 cells were transplanted into nude mice, they produced only

a few tumors that grew after a long latency period and these were comprised of revertant cells. (Rotin *et al.*, 1989) Moreover, only revertants regained the ability to generate tumors, suggesting that Na^+/H^+ exchanger function is necessary for survival as solid tumors develop an acidic microenvironment.

In addition to understanding the importance of these exchangers, developing cells with different levels of function will enable molecular and genetic characterization; which requires amplification or mutation of the gene(s) of interest. (Kuhn *et al.*, 1984) This is achieved through the cloning and sequencing of variants overexpressing or lacking the corresponding gene(s) for exchanger expression. Using this approach Sardet *et al.* (1989) were able to isolate and sequence the cDNA responsible for Na^+/H^+ antiport function.

Flow cytometric sorting has been used successfully to derive variants with different levels of gene expression in other studies. Matsushita *et al.* (1991) generated variants with increased and decreased expression of the surface sialyl-dimeric Le^X (SLX) antigen in the mT-29 colon carcinoma cell line, whilst Johnston *et al.* (1983) were able to obtain cells with increased expression of the dihydrofolate reductase gene in the (CHO) Chinese hamster ovary cell line using fluoresceinated methotrexate.

The purpose of this selection study is two-fold: First, to observe whether variance in pHi of tumor cells within a population can be used as a basis for selecting and propagating cells with different levels of exchanger function. In other words, whether this variation is genotypically

transferrable from generation to generation (i.e. is trait heritable), or just due to random stochastic variation of phenotype within a population of tumor cells. Second, to further the understanding of the Na^+-dependent Cl^-/HCO_3^- exchanger (i.e. for molecular and genetic characterization), I have attempted to derive variant cells selected for increased and decreased levels of Na^+-dependent Cl^-/HCO_3^- exchanger function - for such studies.

Attempts to derive variants with increased and decreased exchanger function have been based on selection for cells with increased and decreased levels of pHi, respectively, from within a population of cells. Those cells in the population that are better able to regulate their internal pH (ie. increased exchanger activity) should possess a higher relative pHi than cells that cannot maintain pHi as well, and *vice versa*. Chambard *et al.* (1986) observed that Chinese hamster lung fibroblast variants lacking the Na^+/H^+ antiport (PS120) were less able to regulate pHi than parental CCL39 cells. The variants possessed a mean value of pHi that was 0.2-0.3 pH units less than parent CCL39 cells, and also required a higher pHe in order to propagate. These results suggest the plausibility of the use of pHi as a discriminating factor to select for various degrees of Na^+-dependent Cl^-/HCO_3^- exchanger operation.

Flow cytometry allows for the rapid assessment of cell characteristics on an individual cell basis; large numbers of cells can be sorted rapidly and repeatedly based on phenotype, with the potential for avoiding mutagens. (Stainano-Coico *et al.*, 1993) Moreover, with BCECF (minimally toxic pH sensitive fluorescent dye) it possible to sort for cells on the basis of different levels of pHi by

gating on various regions of the distribution in pHi values within a population of tumor cells using fluorescence acitivated cell sorting.

The success of the sorting endeavor is dependent on several factors: 1. The fluorescent probe must be non-toxic at the optimal concentration required for resolving differences in pHi, and 2. The accuracy of the pHi measurement, which is based on two components: a. the sensitivity, or the ability of the instrument to detect differences in the means of cell populations normally distributed in pHi; b. and the capacity to resolve subpopulations of different pHi within the measured sample from "background noise". (Musgrove and Hedley, 1990)

BCECF is non-toxic at its optimum concentration of 2μg/mL for up to 2 hours and does not affect clonogenic ability. (refer to section 3.3.4 for my BCECF toxicity experiments as well) (Musgrove *et al.*, 1986, Van Erp *et al.*, 1991) The second requirement is also satisfied. The sensitivity is equivalent to the magnitude of the difference in pHi represented by one channel number (or the slope of the calibration curve). Calibration curves obtained have slopes of ~200-225 channels per pH unit. These values translate to a maximum sensitivity of ~0.004-0.005 pH units, which corroborates with published results. (Boyer and Hedley, 1994) However, in practice, the actual sensitivity is less than the theoretical value (~0.08 pH units) because of the lack of resolution in pHi. (See Chapter Two, section 2.3.5 for estimation of resolution) (Franck *et al.*, 1996)

It was demonstrated previously (in Chapter Two) that there is heterogeneity in pHi within populations of one human and two rodent cell lines. It has been found that in some cell lines the pHi varies as cells progress through the cell cycle. Musgrove *et al.* (1987) observed that in PMC-22 human amelanotic melanoma, CCRF-CEM T-cell leukemia, and L1210 MTXR mouse leukemia, cells were more alkaline in the S, G2, and M phases of the cycle. Cells comprising the upper and lower extremes (10%-20%) of the pHi distribution were analyzed for DNA content using flow cytometry. They observed that cells at low pHi were enriched in G1 DNA content while at high pHi, the proportion of S and G2+M DNA content was increased. In addition to variation in pHi, certain conditions have been correlated with the rate of cell cycle progression, or cell proliferation. It has been found that plating cells in low pHe medium (6.5) led to cytoplasmic acidification and transition to quiescence, or G_1/G_0 arrest. (Taylor and Hodson, 1984, Musgrove *et al.*, 1987) Growth arrest has also been observed in monolayer cultures grown to confluency, probably due to a combination of contact inhibition, acidic pHe, and deprivation of essential nutrients.

Conversely, alkalinization of cells has been observed to induce or increase cell proliferation; for example the exposure of cells to mitogens has been reported to lead to increased pHi and cell proliferation. Moreover, in the absence of mitogens, alkalinization of the cell cytosol has been found to augment the rate of cell turnover. (see Grinstein *et al.*, 1989 for review)

The purpose of my cell-cycle study was two-fold: to observe the influence of cell cycle phase distribution on the distribution in pHi of a murine mammary sarcoma cell line (EMT6), and to

examine any variation in cell cycle distribution in control cells versus cell populations selected on the basis of low and high levels of pHi, and then expanded in culture.

Using dual laser excitation and the fluorescent probes semicarboxyseminapthorhodafluor (SNARF-1) for pH-sensitivity, and Hoechst 33342 for DNA, it is possible to examine cell cycle distribution relative to pHi. Although BCECF was used previously to select for cells with different pHi values, for the purpose of dual laser flow cytometry SNARF-1 is the more desirable stain. Hoechst 33342 fluorescence is measured at 450nm following excitation via a 325nm He-Cd laser. (Durand, 1982, Boyer *et al.*, 1993) Potential problems may arise from the concomitant use of BCECF with Hoechst 33342, since the BCECF absorption spectra indicates maximum absorption at 450-500nm, which may dampen the Hoechst 33342 blue fluorescence emission signal. In contrast, SNARF-1 maximum absorption is at 550-600nm; well above Hoechst 33342 emission fluorescence, thus eliminating the problem of overlap in fluorescence signal. (Fig. 3.1) (Molecular Probes, 1989)

Hoechst 33342 is one of the few stains that selectively and quantitatively binds to DNA in living cells, and thus is useful for selections based on DNA content. (Durand, 1982, Durand and Olive, 1982) Although Hoechst 33342 is a mitotic inhibitor and very high concentrations is toxic to cells, this can be avoided at low concentrations of $\leq 10\mu M$. (Durand and Olive, 1982) Moreover, like BCECF, Hoechst 33342 is retained in the cell for up two hours, which has been the usual duration of the sorting process. SNARF-1 is also non-toxic at its optimal concentration of $5\mu M$.

It is retained well in cells for up to 2 hours, and exhibits good resolution, particularly in the pH range of 7.0-8.0 (0.3 pH units). (Van Erp *et al.*, 91)

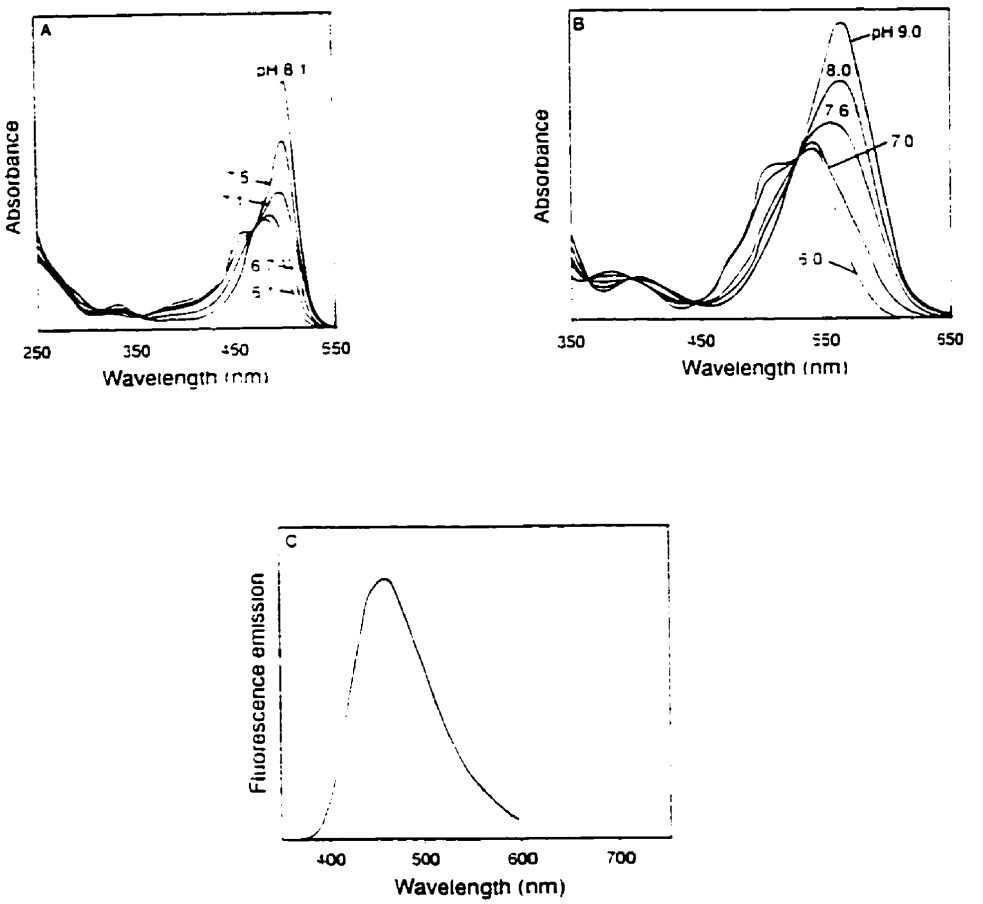

Fig.3.1: Absorption spectra of A) BCECF, B) SNARF-1; emission spectra of C) Hoechst33342. (Molecular Probes, 1996)

In addition to pHi-DNA studies, the cell cycle phase distribution of sorted vs. control EMT6 cells has been compared using the DNA stain propidium iodide. Although the permeabilization of cells

via Triton X-100 detergent is required for staining, propidium iodide has been a popular stain for DNA distribution studies because of the consistent quality of the G_1/G_0 peak resolution. (Taylor and Milthorpe, 1980, Deitch *et al.*, 1982)

3.3 Materials and Methods

3.3.1 Cells:

The experiments were conducted with murine mammary sarcoma cells(EMT6, obtained from Dr. R. Sutherland. Cancer Center for Experimental Therapeutics, Rochester School of Medicine and Dentistry, Rochester, NY). EMT6 cells were utilized in the selection process because they could be studied in suspension and on coverslips. Furthermore, pHi distribution studies revealed the best separation in intracellular pH values; which should facilitate the isolation of different levels of exchanger function.

The maintenance of the cells and the mycoplasma testing method are described in Chapter Two.

3.3.2 Reagents:

Most of the reagents in this study have been previously described in Chapter Two.

SNARF-1, and Hoechst 33342 were acquired from Molecular Probes (Eugene, OR). Stock solutions of SNARF (1mM) and Hoechst 33342 (1mM) were made in 100% DMSO and in distilled H_2O, respectively.

3.3.3 Buffers:

Unless otherwise indicated the following buffers were nominally free of HCO_3^-, as described in Chapter Two: PBS(phosphate buffered saline), K^+ solutions, Na^+ solutions, Na^+ and HCO_3^- solutions.

NMG buffer contained the same compounds as Na^+ solution except that 140mM of NMG was used instead of NaCl. For NH_4Cl-acid load experiments NMG solutions containing 5mM and 15mM of NH_4Cl were used.

Propidium iodide solution was comprised of propidium iodide (250μg/mL) and 0.5% of the cell-lysing detergent Trition X-100 in 4X distilled H_2O.

3.3.4 Instruments Used:

Experiments were conducted using a Perkin Elmer LS3 fluorometer (Perkin Elmer, Mississauga, Ont) equipped with a Xenon lamp and a Coulter Epics Elite Flow Cytometer (Coulter Electronics, Hialeah, FL, USA) equipped with helium-cadmium (He-Cd) (325nm) and argon (488nm) lasers. The He-Cd laser was used to stimulate Hoechst 33342 excitation at 325nm and fluorescence at 450nm was assessed. The argon laser induced SNARF-1 excitation at 488nm and ratiometric fluorescence emission measurements at 580nm and 640nm were conducted. The argon laser was also used to induce BCECF excitation at 488nm, and fluorescence ratio measurements at 525nm and 640nm were performed.

3.3.5 Assessment of BCECF Toxicity as a Function of Time and pHe

Initially, an assessment of the plating efficiency of EMT6 cells stained with BCECF as a function of pHe and time was performed. Approximately 4 X 10^7 cells were centrifuged, resuspended in PBS and stained with 2μg/mL BCECF at 37°C for 30 minutes. Aliquots of approximately 10^6 cells were placed in separate test tubes and re-centrifuged in order to dispose of BCECF dissociated in the external solution. The cells were then suspended in Na^+ and HCO_3^- solution with 10μM EIPA (for Na^+-dependent Cl^-/HCO_3^- exchanger activity) buffered to pHe 7.4, 7.0, 6.6, and 6.2.

After 5, 15, 30, 60, and 120 minutes of exposure, the cells were centrifuged and resuspended in fresh αMEM medium. Approximately 100 cells were plated onto petri dishes and incubated for 8 to 9 days, whereupon clonogenic survival was assessed using the following formula:

$$\text{Relative plating efficiency} = \frac{\text{Average plating efficiency of test sample}}{\text{Average plating efficiency of control}}$$

Control cells were exposed to Na^+ and HCO_3^- solution and EIPA in the presence of 2μg/mL DMSO at the same pHe levels as test samples.

3.3.6 Selection of Cells on the Basis of pHi

Approximately $3X10^7$ to $5X10^7$ cells were used during the two hour duration of the sorting procedure. These cells were initially centrifuged, resuspended in PBS, and stained with BCECF (2μg/mL) at 37°C for 30 minutes. Cells were then re-centrifuged and placed in acidic (pHe 6.6) Na^+ and HCO_3^- solution in the presence of EIPA (10μM) and 10% dialyzed fetal calf serum to make cells dependent on the Na^+-dependent Cl^-/HCO_3^- exchanger. Cells were exposed to a pHe value of 6.6 for the entire duration of the sorting procedure. pHe 6.6 was chosen because for EMT6 cells optimum separation was observed in pHi values with minimum relative toxicity to BCECF exposure for up to two hours. Exposure to BCECF at lower pHe values resulted in greater toxicity, and it was necessary to choose experimental conditions to maximize cell clonogenic ability. Using flow cytometry, cells from the upper and lower 5% regions of the distribution of pHi were selected and grown in selective media. (Fig.3.2)

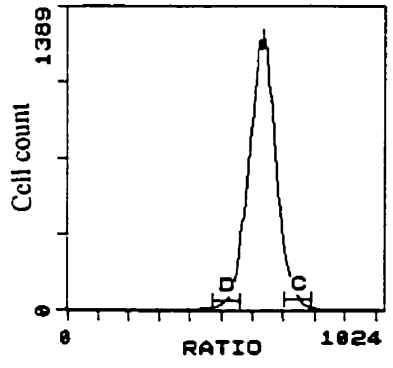

Fig.3.2: Sample pHi distribution of an EMT6 ceil population placed in Na^+ and HCO_3^- buffer and EIPA at pHe 6.6, where the upper 5% (region "c") and lower 5% (region "d") have been selected. Calibration curves were not constructed during the sorting experiments. Instead, cells were sorted after aliquots of the population to be sorted were placed in K^+ buffer (140mM) in the presence of nigericin (10μM) at pHe 7.4 and 6.0 to optimize high and low fluoresence signals.

The cells selected for increased exchanger activity (high pHi) were then grown up in αMEM media in the presence of HCO_3^- at pHe 7.1 (the median pHe of tumors in vivo; this slightly low value should encourage continued function of the exchanger) and pHe 7.4, while those selected for decreased function (low pHi) were placed in medium at pHe 7.4. Selected cells were not able to proliferate at pHe lower than pHe 7.1. When enough cells were obtained, the sorted populations were then resorted under the same conditions.

Control populations were subjected to similar growth conditions as the selected populations, but were not sorted. These controls were essential to associate the variant cells with the specific phenotype of interest.

3.3.7 NH_4Cl-Acid Load Recovery Studies

After each round of selection the activity of the Na^+-dependent Cl^-/HCO_3^- and Na^+/H^+ exchangers of control and sorted populations were quantified directly using NH_4Cl-pulse acidification and fluorometry. Approximately 10^5 cells were seeded onto coverslips, which were positioned in the fluorometer at 30° to the incident beam, and exchanger activity was quantified in a perfusion system. The level of cytoplasmic acidification is dependent on the concentration of external NH_4Cl used. Cells were placed in 5mM or 15mM of NH_4Cl for approximately 30 minutes and incubated at 37°C with 2μg/mL BCECF. Acidification to pHi of approximately 6.8 or 6.6, respectively, was achieved by replacing the solution with NH_4Cl-free NMG buffer. After the

internal pH stabilized, the rate of pHi recovery when NMG was replaced with Na^+ solution (to select for Na^+/H^+ antiport activity), or Na^+ and HCO_3^- solution in the presence of EIPA (for Na^+-dependent Cl^-/HCO_3^- exchange function) was measured. (Fig.3.3)

The quantification of exchanger activity using BCECF and fluorimetry

A. Cells are acidified by replacing NH_4Cl solution with NMG.

B. Exchanger activity is assessed by replacing NMG with one of the following:
1 $NaHCO_3$ + EIPA for Na^+-dependent Cl^-/HCO_3^- exchange.
2 $NaHCO_3$ for both exchanger activity
3 NaCl + EIPA for neither exchanger activity
4 NaCl for Na^+/H^+ exchange.

C. The slope of pHi recovery is a measure of exchanger (non)function.

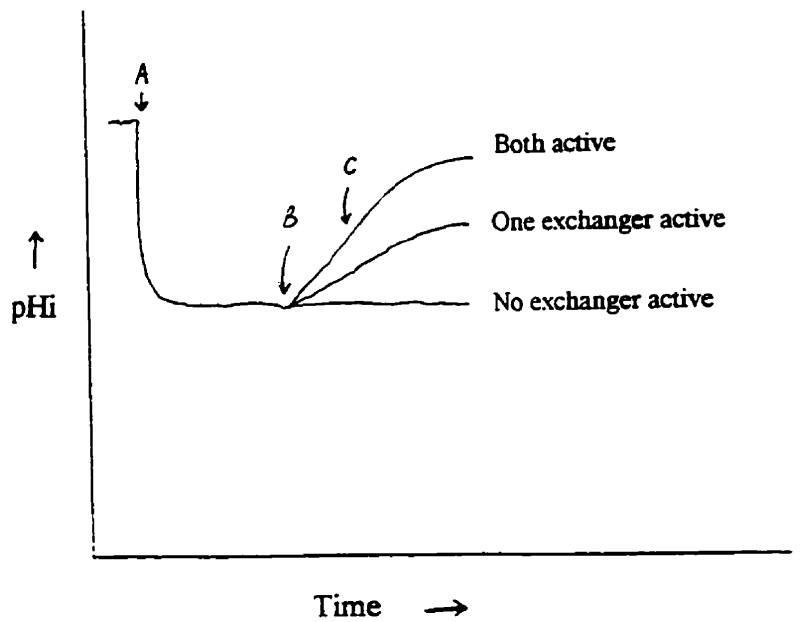

Fig.3.3: Schematic description of NH_4Cl-acid load recovery technique. Diagram is a representation of the data obtained when one (exposure to Na^+ or Na^+ and HCO_3^- buffer in the presence of 10μM EIPA), both (exposure to Na^+ and HCO_3^- buffer), or no exchanger (exposure to Na^+ buffer in the presence of EIPA) is active.

3.3.8 Assessment of Cell Cycle Phase Distribution of EMT6 Cells

Control cells and cells selected and expanded in culture (10^6 cells/mL) were centrifuged, resuspended in PBS containing SNARF-1 (5μM final concentration) and Hoechst 33342 (10μM final concentration) and incubated at 37°C for 30 minutes. The stained cells were subsequently centrifuged and resuspended at $4X10^6$ cells/160μL of PBS with 10% dialyzed fetal calf serum. Approximately 40μL aliquots were then added to 1mL Na^+ and HCO_3^- buffer (with 10μM EIPA to block Na^+/H^+ antiport function) at pHe 6.6. A pHe of 6.6 was used in order to determine whether the pHi of the cells within the population, exposed to sorting conditions, were influenced by cell cycle phase distribution. Subsequently, using dual He-Cd and argon laser flow cytometry, the pHi within a population of EMT6 cells relative to cell cycle phase was examined using the dual parameters of pHi fluorescence ratio and DNA content.

Comparisons of cell cycle distribution were also conducted for sorted-and-expanded vs. control populations utilizing the DNA intercalating stain propidium iodide. Suspensions of 10^6 cells/mL in PBS were exposed to 150μL of propidium iodide containing solution and 20μL of RNase, and stained at room temperature for approximately 30 minutes. Measurements of cell cycle distribution were assessed by measuring propidium iodide fluorescence with argon laser flow cytometry at 640nm emission.

3.4 Results

3.4.1 Assessment of BCECF toxicity as a function of time and pHe

The relative plating efficiency (P.E.) for EMT6 cells exposed to pHe as low as 6.2 for up to 1 hour was ~0.8. At 2 hours of exposure the relative plating efficiency decreased to ~0.7, which was not a significant difference. The level of pHe did not appear to enhance BCECF toxicity in EMT6 cells for up to two hours of exposure. (Fig.3.4)

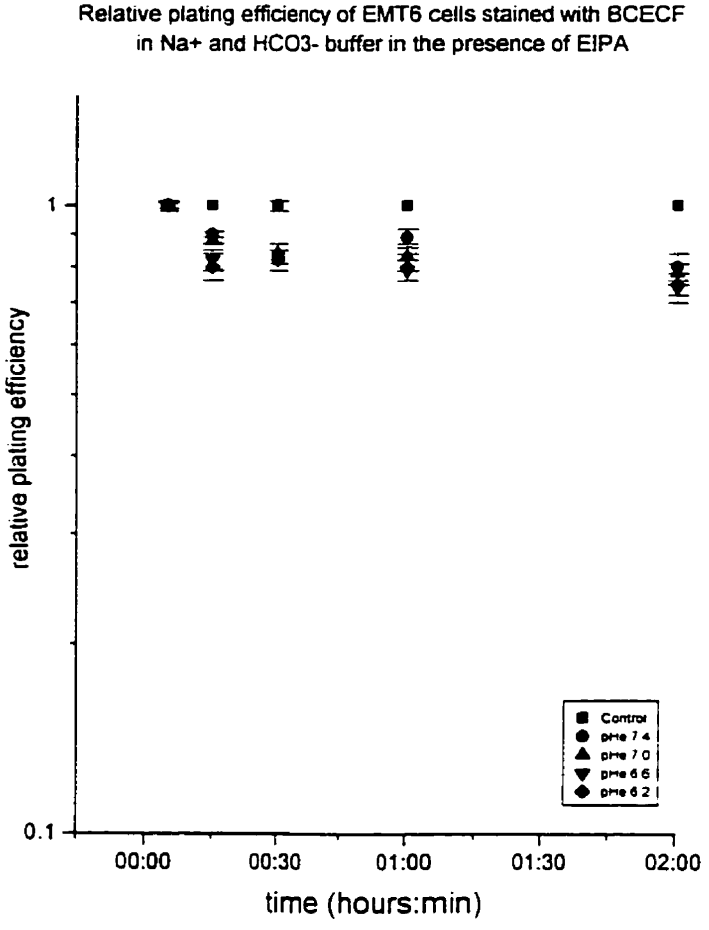

Fig.3.4: Plating efficiency of EMT6 cells exposed to 2μg/mL BCECF and different levels of pHe in the range of 6.2 to 7.4 in Na^+ and HCO_3^- buffer in the presence of 10μM EIPA for up to two hours. (Error bars represent S.E., n=3)

3.4.2 Selection for Increased and Decreased Na^+-dependent Cl^-/HCO_3^- Exchanger Function

The distribution of pHi and the average pHi values of EMT6 cells remained stable during the two hour duration of the sorting process. The average CV value for the distributions during the sorting procedure was ~8.6%. This value was slightly higher than the CVs obtained in the CV vs pHe studies, but this was probably due to the difference in the number of cells analyzed (~1 X 10^4 in CV vs pHe experiments vs. >2 X 10^5 in the sorting procedures) Furthermore, there was no difference in CV between the pHi distribution of cells at successive rounds of selection. (Fig.3.5)

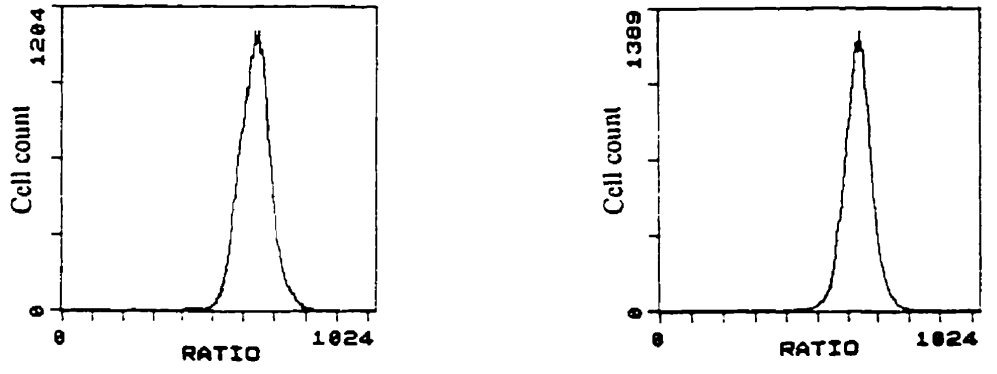

Fig.3.5: Successive pHi distributions of EMT6 cell populations exposed to Na^+ and HCO_3^- buffer and EIPA (10μM) at pHe 6.6 and sorted for increased and decreased levels of pHi. (Left panel: first round of selection, right panel: second round of selection) Calibration curves were not constructed during the sorting experiments. Instead, cells were sorted after aliquots of the population to be sorted were placed in K^+ buffer (140mM) in the presence of nigericin (10μM) at pHe 7.4 and 6.0 to optimize high and low fluoresence signals.

The sorting process seemed to be stressful to the cells. Subpopulations of cells took a relatively long time to grow to near confluency as compared to control populations. Initially when the selected populations of cells were seeded (~1 X 10^6) it was found that they took >5X longer than controls to reach confluency. However, after this initial lag in growth, the sorted populations' doubling times were approximately the same as control cells (~12 hours). It was impossible to perform immediate reanalyses of selected subpopulations because of dye leakage and decrease in cell viability (very dim cells with increased side scatter and decreased forward scatter); making it difficult to distinguish between cells. The selection process was carried through for two generations.

3.4.3 Acid Load Recovery Studies of Control and Sorted Populations

In acid load recovery studies using 5mM and 15mM of NH_4Cl (ie. from different levels of pHi) populations selected for increased and decreased levels of pHi exhibited slightly higher or lower Na^+-dependent Cl^-/HCO_3^- exchanger activity, respectively, than controls. (Table.3.1)

However, the differences between high and low selected populations from each other and from controls were not significant. The average Na^+-dependent Cl^-/HCO_3^- exchanger recovery rates were ~3.7 X 10^{-3} pH units/s after exposure to 15mM NH_4Cl and ~5.7 X 10^{-3} pH units/s after exposure to 5mM NH_4Cl. This is consistent with greater activity of the exchanger at pHi values just below physiological. In contrast, average Na^+/H^+ antiport recovery rates were greater in general (~8.6 X 10^{-3} pH units/s after exposure to 15mM NH_4Cl and ~1.1 X10^{-2} pH units/s after

exposure to 5mM NH_4Cl), but there was no difference between control and sorted cells; which indicates that Na^+/H^+ exchanger function was not affected by our selection conditions. In addition, there was no difference in exchanger function between cells selected for high exchanger function grown at pHe 7.1 vs. 7.4.

NH_4Cl-Acidification Analysis (Rate of Recovery) of Na^+-dependent Cl^-/HCO_3^- Exchanger and Na^+/H^+ Antiport Activity in EMT6 Control and Sorted-and-Expanded Populations (pH units/s; n=5)

	Na^+-dependent Cl^-/HCO_3^- Activity		Na^+/H^+ Exchange Activity	
Condition	NH_4Cl 15mM	NH_4Cl 5mM	NH_4Cl 15mM	NH_4Cl 5mM
Control (7.4)	$3.4(\pm0.8)X10^{-3}$	$5.7(\pm0.5)X10^{-3}$	$7.9(\pm0.5)X10^{-3}$	$15.0(\pm1.7)X10^{-3}$
Control (7.1)	$4.1(\pm0.9)X10^{-3}$	$5.2(\pm0.9)X10^{-3}$	$7.5(\pm1.2)X10^{-3}$	$11.0(\pm1.6)X10^{-3}$
Low Function I (7.4)	$2.9(\pm0.2)X10^{-3}$	$3.2(\pm0.3)X10^{-3}$	$8.7(\pm0.9)X10^{-3}$	$10.0(\pm1.4)X10^{-3}$
Low Function II (7.4)	$2.5(\pm0.7)X10^{-3}$	$4.3(\pm0.5)X10^{-3}$	$8.0(\pm0.8)X10^{-3}$	$10.0(\pm1.0)X10^{-3}$
High Function I (7.1)	$5.8(\pm1.4)X10^{-3}$	$5.4(\pm1.0)X10^{-3}$	$10.0(\pm1.7)X10^{-3}$	$12.0(\pm1.4)X10^{-3}$
High Function I (7.4)	$3.1(\pm0.4)X10^{-3}$	$4.8(\pm0.5)X10^{-3}$	$9.0(\pm1.5)X10^{-3}$	$10.0(\pm1.8)X10^{-3}$
High Function II(7.4)	$4.2(\pm0.8)X10^{-3}$	$6.4(\pm1.4)X10^{-3}$	$9.0(\pm1.2)X10^{-3}$	$11.0(\pm2.2)X10^{-3}$

Table 3.1: Rate of recovery after exposure to 15mM or 5mM NH_4Cl of control and cells selected for increased (High function) and decreased (Low function) Na^+-dependent Cl^-/HCO_3^- exchanger activity and expanded in culture. I and II indicate generations of selected cells that were obtained. EMT6 cells were subjected to the following conditions: 1) Na^+ and HCO_3^- buffer in the presence of 10μM EIPA to select for Na^+-dependent Cl^-/HCO_3^- exchanger activity. 2) Na^+ buffer to select for Na^+/H^+ antiport function.

3.4.4 Cell Cycle Distribution Studies

EMT6 dual parameter pHi vs. DNA distribution studies of control cells and cells selected-and-expanded in culture showed that the pHi remained constant in cells at different phases of the cell cycle. (Fig.3.6)

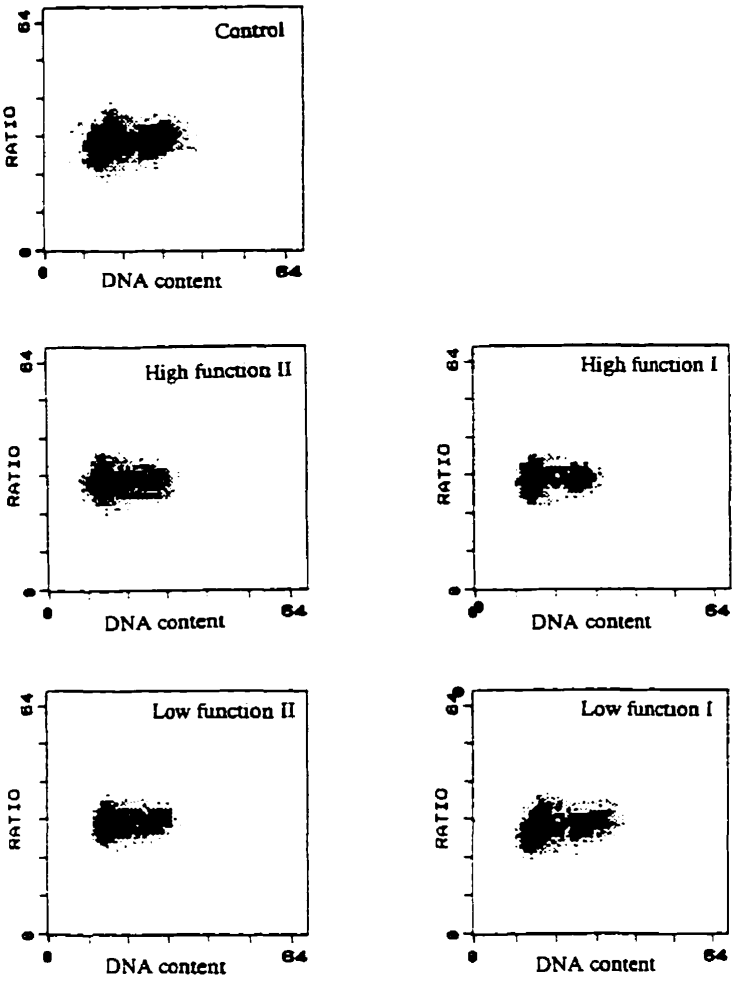

Fig.3.6: Comparisons of pHi (ratio) with DNA content of control cells and cells selected for increased (High) and decreased (Low) levels of pHi and expanded in culture. EMT6 cells were stained with 5μM SNARF-1 and 10μM Hoechst 33342 in Na^+ and HCO_3^- buffer and EIPA (10μM) at pHe 6.6. I and II indicate the different selection generations that were analyzed. With Hoechst 33342 staining it was not possible to distinguish between S and G2+M phases of the cell cycle (see Discussion) so G1, S, and G2+M fractions could not be determined. Calibration curves were not generated for these experiments (performed three times). Instead, aliquots of the sample populations were exposed to K^+ buffer (140mM) in the presence of nigericin (10μM) at pHe 7.4 and 6.0 to optimize high and low fluoresence signals.

Moreover, when the pHi-DNA distribution analyses were conducted on populations of gated (but unsorted) cells exposed to the sorting conditions, the upper and lower ~5% gated regions of the distribution in pHi (subpopulations with high and low pHi relative to majority of cell population) showed similar cell cycle phase distributions as the ungated population of cells. (Fig.3.7)

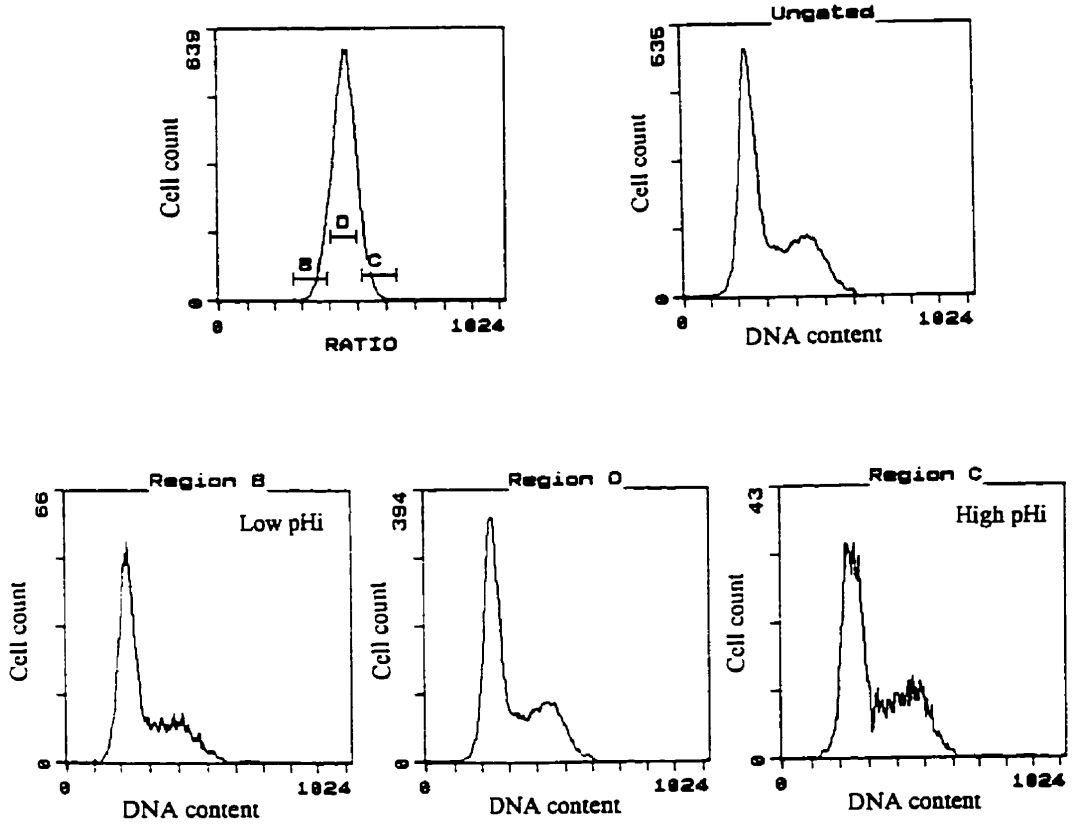

Fig.3.7: Respective DNA distributions of ungated, upper and lower (~5%), and middle gated regions of a pHi distribution of EMT6 cells stained with 5μM SNARF-1 and 10μM Hoechst 33342 and exposed to Na^+ and HCO_3^- buffer and EIPA at pHe 6.6. With Hoechst 33342 staining it was not possible to distinguish between S and G2+M phases of the cell cycle (see Discussion), so G1, S, and G2+M fractions could not be determined. Calibration curves were not generated for these experiments (performed three times). Instead, aliquots of the sample populations were exposed to K^+ buffer (140mM) in the presence of nigericin (10μM) at pHe 7.4 and 6.0 to optimize high and low fluoresence signals.

Initially I had attempted to analyze pHi-DNA distributions in control cells and subpopulations immediately after selection for different levels of pHi. However, this was not possible because it was difficult to distinguish between cells within the sorted populations due to dye leakage, and decreased cell viability. As a result, only control and sorted-and-expanded populations could be compared.

Propidium iodide examinations of control and sorted populations of EMT6 cells revealed no difference in the distribution of cell cycle. (Fig.3.8) In addition, the S-phase fraction (%) of control and sorted populations were similar, with values ranging from approximately 40% to 43%. (Table 3.2) Furthermore, there was little difference in G1 between control and sorted EMT6 cells, with values ranging from 39% to 45%. (CV values were ~4.6)

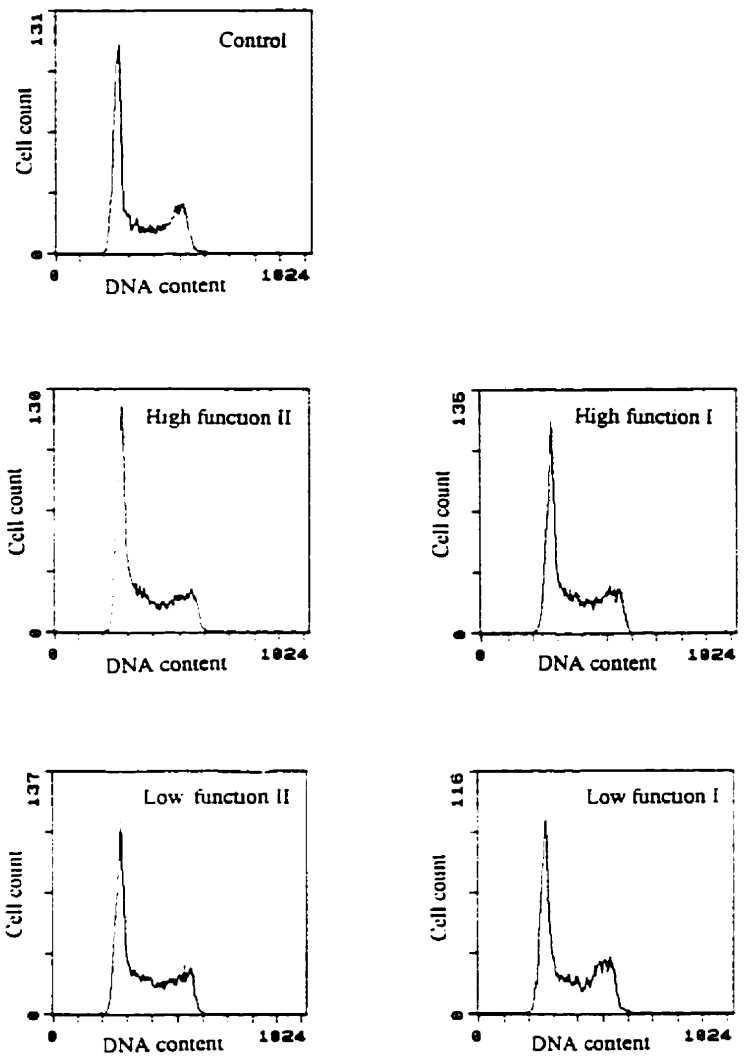

Fig.3.8: Propidium iodide DNA distributions of EMT6 cells selected for increased (High function) and decreased (Low function) levels of pHi and expanded in culture. I and II indicate the different selection generations that were analyzed.

Propidium iodide DNA distribution analyses of control and sorted populations of EMT6 cells (n=3)

	G1 fraction (%)		G2-M fraction (%)		S fraction (%)	
Cell condition	Mean	S.E.	Mean	S.E.	Mean	S.E.
Control grown at 7.4	41.1	3.0	17.5	0.9	41.4	2.8
Control grown at 7.1	45.2	3.0	15.0	1.4	39.8	1.6
High II grown at 7.4	40.9	3.0	14.9	3.7	44.2	2.3
High I grown at 7.4	44.3	3.0	12.9	2.7	42.8	4.6
High grown at 7.1	41.7	4.5	15.0	4.0	43.2	5.0
Low II grown at 7.4	38.7	1.4	18.1	1.7	41.0	1.7
Low I grown at 7.4	38.7	3.2	20.4	1.0	40.9	3.3

Table 3.2: Comparisons of G1, G2-M, and S-phase fraction (%) of EMT6 cells selected for increased(High) and decreased (Low) levels of pHi and expanded in culture. Cells were analyzed with the propidium iodide staining technique. I and II indicate the different selection generations that were analyzed.

3.5 Discussion

Acid load recovery studies showed that both exchangers were active at pHi values achieved by NH_4Cl exposure, which suggests that this activity at least allowed for the possibility of selection. Under acute acidification conditions cells exhibited greater Na^+/H^+ antiport activity which was not observed in my previous pHi vs pHe studies. One possibility for this discrepancy is the acute acidification method that was utilized in these studies; acidification to pHi levels as low as ~6.6 (5mM NH_4Cl) and to ~6.4 (15mM NH_4Cl) was observed - which is in the reported range of greatest Na^+/H^+ transport function in EMT6 cells. (Boyer *et al.*, 1993)

The derivation of variants is based on the genetic or epigenetic transferrance of a trait from generation to generation. Our current experiments failed to select variants using pHi to discriminate for different levels of Na^+-dependent Cl^-/HCO_3^- exchanger operation. With our selection process, it was expected that the separation in pHi between EMT6 cells sorted for higher and lower relative pHi should increase with each round of selection, however this was not observed. Nevertheless, only two rounds (generations) of selection were attempted due to technical problems (see section 3.6). Given the low rate of variants/mutants within a population of cells, two levels of selection might not have been sufficient to isolate for different levels of phenotype in the subpopulations. However, previous groups were able to observe different levels of expression in a phenotype after one round of selection. (Johnston *et al.*, 1983, Matsushita *et al.*, 1991) This implies that the number of rounds of selection is not so much a factor in observing different levels of function, as it is in the maintenance of stable variants expressing different levels of activity. Subsequently, selection was attempted with CHO cells and MCF-7 cells (data not shown). MCF-7 cells were chosen for their versatility for coverslip attachment and cell suspension methods, and for a human - murine cell comparison. CHO cells were also studied because of their functionally haploid nature, as compared to aneuploid EMT6 and MCF-7 cells. (Siminovitch and Thompson, 1978) This may lead to a higher probability of variants in the population since loss of function may occur after inactivation of a single gene. In the above cell lines it was also observed that there was no difference in level of exchanger function between control and sorted populations. This suggests that the variation in level of Na^+-dependent Cl^-/HCO_3^- exchange activity is likely a result of random stochastic expression in EMT6, CHO, and MCF-7 cells.

These results do not rule out the possibilty of the genetic determination of exchanger expression/function. The genetic influence on the level of Na^+-dependent Cl^-/HCO_3^- exchanger activity may be more specifically assessed by mutagenesis analysis. Through the use of mutagens it is possible to observe the following: 1. Gene amplification (eg. using hydroxyurea) analyses can be used to monitor the subsequent effect on increased exchanger expression. 2. Mutation/deletion (eg. using ethane methane sulfonate) analyses can be used to isolate specific regions of the genome responsible for exchanger expression, since loss/mutation of the region will lead to loss/change in phenotype. However, with mutagens it is impossible to control other mutations in phenotype - some of which may be undesirable (such as the loss of tumor-forming ability or K^+/Na^+ exchange activity).

There have been no documented studies aimed at deriving variants with varied Na^+-dependent Cl^-/HCO_3^- exchanger activity. If we assume that this is a reflection of previously unsuccessful attempts at selection, prior attempts might have been difficult for the following reasons: Accounts of successful variant selection using flow cytometry used a more direct approach to antigen selection. For instance, Matsushita *et al.* (1991) used monoclonal antibodies specific for the SLX antigen and Johnston *et al.* (1983) used fluoresceinated methotrexate - which binds quantitatively to dihydrofolate reductase. In comparison, although pHi has been shown to influence Na^+-dependent Cl^-/HCO_3^- transporter activity, it is not a specific discriminator of this particular exchanger's function. A more desirable method might be to use a fluorescent stain attached to DIDS or to other drugs/markers that inhibit the exchanger itself (and therefore may be

bound to it). However, there exists no known drug that binds exclusively to the Na^+-dependent Cl^-/HCO_3^- exchanger. Second, the selection procedures might have been ineffective due to problems such as those outlined in section 3.6.

Other possibilities for the derivation of variants are modifications of Pouyssegur's methods to select for increased and decreased Na^+/H^+ antiport function. A variation on the proton-suicide technique for the selection of cells with decreased Na^+-dependent Cl^-/HCO_3^- exchanger activity is to place cells in high Cl^-, and HCO_3^- and Na^+ free buffer at acidic pHe (5.5) in the presence of EIPA. Assuming that the exchanger can operate in reverse, those cells with little or no Na^+-dependent Cl^-/HCO_3^- transporter function will only survive because their cytoplasm will not acidify.

Derivation of variants with increased Na^+-dependent Cl^-/HCO_3^- exchanger activity might also be achieved by placing cells in successively more acidic medium in the presence of EIPA, or by exposing cells to a prolonged acid load (in the presence of EIPA) in Na^+ and HCO_3^- buffer, and increasing concentrations of DIDS. Derivation of variants from incubation in acidic medium has been difficult due to the problems with achieving an acidic environment that will encourage increased activity without affecting the proliferation or clonogenic activity of the cultured cells. In addition, exposure to DIDS may result in additional altered characteristics. Franchi *et al.* (1986) were successful in obtaining clones with increased Na^+/H^+ antiporter activity by exposing cells to a prolonged acid load. However this method required the presence of increasing concentrations of (5-N-methyl, N-propyl)amiloride (MPA) (to block Na^+/H^+ exchange), or to

decreasing concentrations of external Na^+; this strategy resulted in variants with increased exchanger activity, but different affinities for MPA or external Na^+ than controls.

Preliminary incubation of CHO, EMT6, and MCF-7 cells at pHe 7.0-7.1 did not result in increased levels of exchanger activity (data not shown). In addition, prolonged incubation at more acidic pHe has resulted in the induction of G_1/G_0 growth arrest. (Taylor and Hodson, 1984, Musgrove *et al.*, 1987) For example, although Boyer *et al.* (1993) observed increased exchanger activity in EMT6 and MGH-U1 cells grown in monolayer at pHe 6.5 for 18 hours, longer exposure led to cytotoxicity.

Preliminary experiments (repeated three times) were conducted where EMT6 cells were exposed to Na^+ and HCO_3^- buffer with either: low $[Na^+]e$ (70mM or 20mM) and $[HCO_3^-]e$ (5mM); or to Na^+ and HCO_3^- buffer with increasing concentrations of DIDS (50μM, 100μM, and 200μM). The experiments were unsuccessful due to cytotoxicity (data not shown) which was observed when cells were exposed to such stressful conditions for a period of 1 to 18 hours. Continued experimentation was abandoned because of the limitations of time, however further studies at shorter exposure durations (< 1 hour), and gradual and more frequent adjustments to buffer and DIDS concentrations may produce more useful results.

DNA analyses using propidium iodide indicate that control and sorted-and-expanded populations of EMT6 cells have similar cell cycle phase distributions. Since control and sorted populations exhibited similar doubling times, it is expected that their cell cycle distributions would be fairly

similar. In addition, these comparisons were made between control cells and sorted populations that were expanded in culture. As a result, any immediate differences or changes in cell cycle distribution between control and sorted populations after the sorting procedure could not be assessed.

Dual parameter pHi vs. DNA distribution studies of EMT6 cells showed that the level of pHi remained constant throughout the cell cycle. However, this observation must be regarded as inconclusive because of the difficulty in distinguishing populations in S vs. G2-M phase of the cell cycle with Hoechst 33342 staining as compared to staining with propidium iodide, which precluded relative proportion analyses. (Fig.3.9)

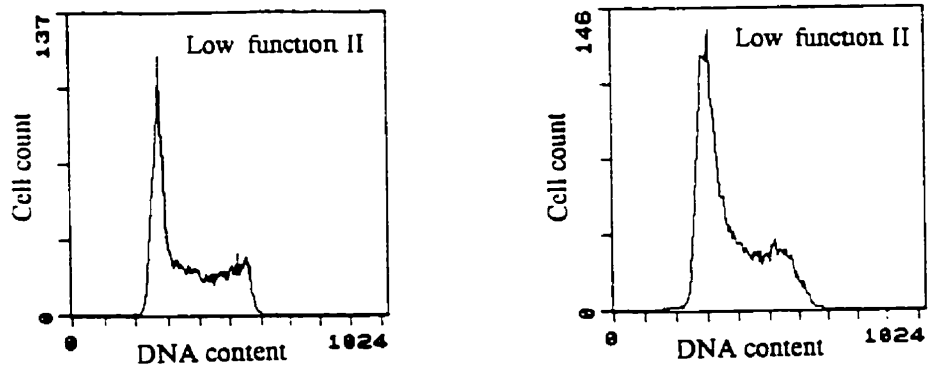

Fig.3.9: Comparison of DNA distributions of EMT6 cells using propidium iodide (left panel) or Hoechst 33342 (right panel) staining technique. For the latter procedure, cells were exposed to Na^+ and HCO_3^- buffer and 10μM EIPA at pHe 6.6.

Nevertheless, I consistently found that the pHi of the cells in G1 and S-G2-M composite phases remained constant. Furthermore, cell cycle comparisons of subpopulations gated for the upper and lower regions of the pHi distribution suggest that the variation in pHi within the population of cells is not a reflection of cells in different phases of the cell cycle. Although there has been documentation of variation in pHi with cell cycle progression (increase in pHi in cells undergoing S-phase DNA doubling), this has only been observed in a few other cell lines. (Musgrove *et al.*, 1987)

Further testing of my results is necessary for confirmation. Although Hoechst 33342 has been assumed to bind quantitatively to DNA, in actuality it binds selectively to AT sites - in particular to groups of 4-5 consecutive base pairs. This implies that staining is dependent on both the AT:CG ratio and on the length of the AT and CG segments in the DNA molecule. (Larsen, 1992) A possible improvement to the pHi-DNA study is the concomitant use of a CG selective dye such as mithramycin or 7-amino actinomycin. The use of the latter fluorescent stain may be simpler because it can be excited at 488nm, thus eliminating further complications of a three laser alignment - in addition to triple parameter analysis.

3.6 Technical Difficulties

I encountered considerable difficulties during the selection process. The instrument did not always exhibit stable resonance (consistent crystal vibration to allow for uniform formation of droplets containing cells); slight perturbations in fluidics (flow of sheath and cell suspension

through system) or crystal frequency - which could neither be anticipated nor controlled - would completely disrupt the sorting procedure. The original bimorph screw mount assembly for the crystal was replaced with a more stationary stainless steel sort oscillator, but this improved resonance only slightly since the new contraption did not preclude the influence of the non-uniform resonance of the casing on the droplet frequency.

In conclusion, pHi distribution does not appear to be an effective approach to select for increased and decreased levels of Na^+-dependent Cl^-/HCO_3^- exchanger activity. In addition, the cell cycle does not seem to influence the level of pHi within populations of EMT6 cells. Although propidium iodide DNA analyses reveal that control and selected cells possessed similar cell cycle distributions, this is expected since the sorted populations were allowed to expand (and adapt) in culture.

3.7 References

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4.1 Overall Conclusions and Future Work

My studies reveal that pHi decreased in cells placed at low pHe, and that the level of pHi was cell line dependent. In EMT6, CHO, and MCF-7 cell lines, only the Na^+-dependent Cl^-/HCO_3^- exchanger influenced the pHi within the experimental conditions of pHi 6.8-7.3. Moreover, I observed that there is heterogeneity in pHi within populations of the above cell lines. Cells with Na^+-dependent Cl^-/HCO_3^- exchanger activity exhibited less variation in pHi, indicating that these cells were able to regulate cytoplasmic pH more effectively, than when the Na^+/H^+ antiport was operating. This suggests that under these conditions, the Na^+-dependent Cl^-/HCO_3^- exchanger is more dominant in pHi regulation. This also supports the suggestion that within the acidic microenvironment of tumors (mean ~6.9, range 5.8-7.7), the Na^+-dependent Cl^-/HCO_3^- exchanger may be more active in pHi regulation.

Subsequent selections for increased and decreased levels of Na^+-dependent Cl^-/HCO_3^- exchanger function (pHi) in all three cell lines suggest that the expression of this exchanger in an unperturbed population is due mainly to random stochastic variation. The use of pHi to discriminate different levels of exchanger function was indirect. Given the low rate of variants/mutants within a population of cells - a more direct selection approach is probably required. A possible improvement to the technique is to use specific fluoresceinated drugs/markers to inhibit the Na^+-dependent Cl^-/HCO_3^- exchanger.

Finally, it was observed in the EMT6 cell line that sorted-and-expanded populations possessed similar cell cycle distributions as control cells. In addition, increased and decreased levels of pHi of cells within a population did not reflect differences in cell cycle. This suggests that the distribution in pHi, under these artificial conditions, did not appear to be cell cycle dependent. However, since the cells selected for increased and decreased levels of pHi were expanded in culture, any possible differences upon sorting could not be assessed.

In order to further our understanding of the Na^+-dependent Cl^-/HCO_3^- exchanger it is imperative to observe its behavior, and to isolate the molecule itself. The development/discovery of inhibitors that are specific for the Na^+-dependent Cl^-/HCO_3^- transporter is important for the following reasons:

1) it will be possible to study the effects on the Na^+-dependent Cl^-/HCO_3^- exchanger alone, which could elucidate stoichiometry of exchange activity.

2) quantitative cytokinetic binding analyses will elucidate whether heterogeneity in function reflects level of expression or concentration of exchangers.

3) direct fluoresceinated inhibitors may be used to quantitatively select for variation in exchanger function/expression using flow cytometry.

4) variable binding analyses will reveal the effects of mutation (eg. overexpression) on the level of Na^+-dependent Cl^-/HCO_3^- exchanger function.

The molecular and genetic characterization of the Na^+/H^+ antiport was made possible through the use of variant cell lines with absent and increased expression. Variants with increased and

decreased expression of Na^+-dependent Cl^-/HCO_3^- transporter activity would be useful for molecular and genetic characterization, and for studies including: 1. sensitivity to perturbations in microenvironmental conditions (*in vitro*) such as acidic pHe, Na^+, HCO_3^-, and Cl^-; 2. Tumor-forming ability of variant and revertant cells when implanted into mice.

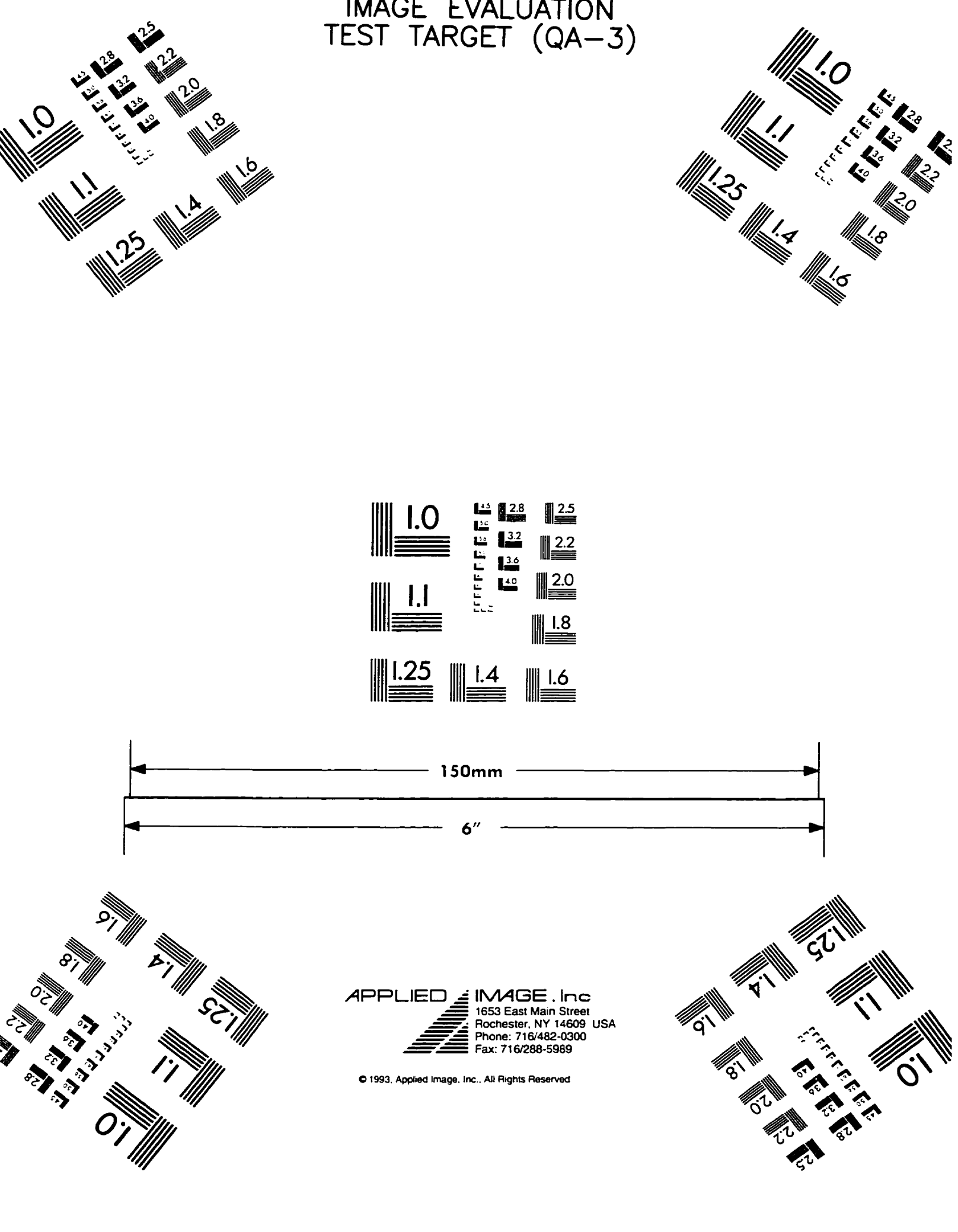
It may be possible to derive variants with increased and decreased Na^+-dependent Cl^-/HCO_3^- exchanger function by expanding clones of selected cells following exposure to mutagens. However, additional changes in phenotype (which may include the loss of tumor forming ability, K^+/Na^+ transport) from mutagenesis cannot be controlled or entirely assessed.

Alternative methods of selection to pursue are: the use of an adaptation from the proton-suicide technique for the selection of cells with decreased Na^+-dependent Cl^-/HCO_3^- exchanger activity by placing cells in acidic media (pHe 5 5) in high Cl^-, and HCO_3^- and Na^+ free buffer in the presence of EIPA. Derivation of variants with increased Na^+-dependent Cl^-/HCO_3^- exchanger activity may also be possible by placing cells in successively more acidic medium in the presence of EIPA, or by exposing cells to a prolonged acid load (in the presence of EIPA) to Na^+ and HCO_3^- buffer, and increasing concentrations of DIDS.

From the perspective of molecular biology, the cDNA encoding the Na^+-dependent Cl^-/HCO_3^- exchanger may be obtained from knowledge of the already characterized Na^+-independent Cl^-/HCO_3^- exchanger. The cDNA and amino acid sequence for the Na^+-independent anion exchange molecule (band 3) is known for mice, rats, and humans. The differential selectivity of the Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchangers to certain drugs (see Chapter One, section 1.6 D) suggests that the two exchange molecules are separate entities. Nevertheless, the two

exchangers exhibit sensitivity to DIDS inhibition and transport the same anions, although in opposite directions. This suggests that the Na^+-dependent and independent Cl^-/HCO_3^- exchanger molecules may share some sequence homology. In the Na^+-independent anion exchange molecule the C-terminal membrane-embedded structural domain is known to mediate Cl^-/HCO_3^- exchange. In addition, evidence suggests that lysine regions between the N-terminal and C-terminal domains possess DIDS binding sites. (Alper, 1991) Therefore, it may be possible to clone and sequence the Na^+-dependent Cl^-/HCO_3^- exchange molecule through hybridization screening with the known cDNA of the Na^+-independent anion exchanger.

Although I was unsuccessful in deriving variants with increased and decreased Na^+-dependent Cl^-/HCO_3^- exchanger expression, it is hoped that the information presented and suggested in this thesis will inspire further work for future success.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150mm
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