

# Influence of pH on the uptake of 5-fluorouracil into isolated tumour cells

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**Summary** To investigate the possible dependence of 5-fluorouracil (5FU) uptake in tumours on the intra- ( $pH_i$ ) and extracellular ( $pH_e$ ) pH, a pH gradient ( $\Delta pH$ ) was imposed across the plasma membrane of ascites tumour cells in vitro, similar to that known to occur in some solid tumours in vivo, by incubation in media of  $pH_o$  5–8. A  $\geq 2:1$  (intracellular/extracellular) accumulation of radiolabelled 5FU occurred after 5 min incubation of the cells with 0.5 mM 5FU at  $pH_o$  of 5.0, 5.5 or 6.0. 5FU metabolism is slow under these conditions, and 5FU uptake was not affected by longer incubations up to 20 min, nor by the absence of a sodium gradient.  $pH_i$  was estimated from the distribution of the weak acid, 5,5-dimethyl-2,4-oxazolodione ( $[^{14}C]DMO$ ) across the cell membrane. There was significant correlation between the intracellular/extracellular 5FU ratio and  $pH_o$  (from  $pH_o$  6–8),  $\Delta pH$  and  $pH_i$  ( $P < 0.02$ ). Similar results were obtained with HT29 cells. Incubation with a drug that made plasma membranes permeable to  $H^+$  significantly decreased 5FU uptake in Lettre cells. The co-transport of 5FU may occur on a proton symport using the proton motive force of the  $\Delta pH$ .

**Keywords:** 5-fluorouracil; intracellular pH; extracellular pH; ascites tumour cells; pH gradient

5-Fluorouracil (5FU) has been in use as an anti-cancer agent for more than three decades since its synthesis in 1957 by Heidelberger et al. Nowadays, it is predominantly used to treat solid tumours such as colon and breast cancers and occasionally for head and neck, and lung cancers, and adjuvant treatments of potential micrometastases, either alone or in combination with other drugs such as interferon, methotrexate or leucovorin. 5FU may be anabolized to cytotoxic nucleotides or deactivated by catabolism, an event occurring predominantly in the liver, but also in other tissues and some tumours (Naguib et al, 1985). 5FU metabolism has been extensively studied by  $^{19}F$  magnetic resonance spectroscopy (MRS) in cultured cells, and in vivo in tumour-bearing animals and patients (for review see Findlay and Leach, 1994). 5FU cytotoxicity is caused by irreversible inhibition of thymidylate synthase (TS) via the generation of the anabolite 5-fluoro-2-deoxyuridine monophosphate (FdUMP) leading to an inhibition of DNA synthesis, and by incorporation of 5-fluorouridine-5-triphosphate (FUTP) into RNA (FU-RNA). The degree of sensitivity to either, or both, of these cytotoxic nucleotides is tissue dependent (Heidelberger et al, 1983). TS inhibition tends to be favoured by prolonged exposure of low 5FU concentrations (approximately 15  $\mu M$ ) for several days, whereas FU-RNA formation tends to be favoured by brief exposures to high concentrations (1 mM) for a few hours (see Aschele et al, 1992). The intracellular concentrations of 5FU and its metabolites are also dependent on the transport and uptake of 5FU into the target cells (Heidelberger et al, 1983).

$^{19}F$ -MRS studies have shown that 5FU appears to be retained longer in tumours than in normal tissues (Presant et al, 1990;

Guerquin-Kern et al, 1991; Findlay et al, 1993). Enhanced retention has also been shown to be significantly associated with response (Presant et al, 1990; Findlay et al, 1993), perhaps because higher concentrations of 5FU will sustain its anti-tumour effects by favouring the lasting presence of toxic metabolites at target tissue sites (Peters et al, 1993). The elimination half-lives ( $t_{1/2}$ ) of 5FU in the VX2 tumour of the rabbit (Wolf et al, 1990), and the Walker 256 adenocarcinoma of the rat (El-Tahtawy and Wolf, 1991), were found to be about 1 h (63–73.2 and 42.2–59.4 min respectively), and greatly exceed the  $t_{1/2}$  of 5FU in rat plasma (Au et al, 1983), which is similar to that reported in humans (5–15 min) (Cohen et al, 1982). Presant et al (1994) found that the response of patients to 5FU could be predicted from the rate of loss of tumour 5FU signal, measured by  $^{19}F$ -MRS. These observations suggest that elucidation of the mechanisms by which 5FU accumulates in tumours could be of clinical significance as they may aid the development of more rational combination chemotherapy.

The pH of tumours measured non-invasively with  $^{31}P$ -MRS has been shown, in most circumstances, to represent intracellular pH ( $pH_i$ ) (Stubbs et al, 1992), whereas microelectrode measurements mainly sample extracellular pH ( $pH_e$ ) (for review see Vaupel et al, 1989). Overall, MRS measurements show that tumours and normal tissues have close to neutral  $pH_i$  values (6.9–7.4) (Griffiths, 1991), whereas  $pH_e$  values for tumours are more acidic than  $pH_i$  (by about 0.3–0.5 pH units).  $pH_e$  values for normal tissues are on the alkaline side of  $pH_i$  with a range from 7.2 to 7.6. Thus, the pH gradient across the membrane of normal cells is from (relatively) acid intracellularly to alkaline extracellularly. In contrast, in tumours the pH gradient is negative, i.e. the reverse of that in control tissues. The acidic  $pH_e$  in tumours is probably due to the high glycolytic rate associated with tumours and the subsequent extrusion of cellular acids.

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A  $^{19}\text{F}$ -MRS study by Guerquin-Kern et al (1991) showed that the  $t_{1/2}$  of 5FU elimination was 2.5-fold longer at  $\text{pH}_i < 6.9$  than at  $\text{pH}_i 7.3$  in a rat fibrosarcoma. A glucose-induced reduction can lead to an even greater decrease in  $\text{pH}_c$  (see Gerweck et al, 1991), thus causing an increase in the negative pH gradient, and it is possible, therefore, that the observed trapping of 5FU in tumours is related to the difference between  $\text{pH}_i$  and  $\text{pH}_c$  across the tumour cell membrane. The aim of the present study was to try to relate the distribution of radiolabelled 5FU to the distribution of  $\text{H}^+$  (referred to as the pH gradient;  $\Delta\text{pH}$ ) across the cell membrane of Lettre cells (a line of mouse Ehrlich ascites cells), and human HT29 adenocarcinoma cells. This was carried out by varying  $\text{pH}_c$  values and assessing the induced  $\text{pH}_i$  from the distribution of the labelled weak acid, 5,5-dimethyl-2,4-oxazolidione (DMO) across the membrane of the cells. Cells were incubated with 5FU at concentrations of 0.1–0.5 mM, which were in the range of peak plasma concentrations measured in mice (Hill and Bibby, 1994) and patients in the clinic (Findlay et al, 1996), and would not be rate limiting for transport (Yamamoto et al, 1981; Heidelberger et al, 1983).

## MATERIALS AND METHODS

### Drugs and chemicals

5FU was obtained as the sodium salt in water from David Bull Laboratories (Warwick, UK). 5FU-6- $^3\text{H}$  (spec. act. 20 Ci  $\text{mmol}^{-1}$ ) was purchased from Sigma Chemical Company (Poole, Dorset, UK) and [ $^{14}\text{C}$ ]DMO from Amersham International (Amersham, UK). HP/b scintillant fluid was obtained from Beckman, and oil (2:1 of di-*n* butyl phthalate and dinonyl phthalate) from BDH chemicals, Poole, UK, and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) from Sigma.

### Production of Lettre cells

White Swiss TO male mice weighing approximately 30 g were injected intraperitoneally with 0.1 ml of  $10^8$  cells  $\text{ml}^{-1}$  that grew as ascites. After 7–10 days, the cells were removed from the peritoneal cavity and suspended in Hepes-buffered saline (HBS) containing approximately 100  $\mu\text{l}$  of heparin. The suspension was centrifuged and the pellet was resuspended in isotonic saline solution to 30% v/v (c.  $3 \times 10^8$  cells  $\text{ml}^{-1}$ , i.e. 300 mg of cells). All experiments were conducted at a room temperature of 20–22°C.

### Measurement of $\text{pH}_i$ using the [ $^{14}\text{C}$ ]DMO distribution method and [ $^3\text{H}$ ]5FU distribution across cell plasma membranes

Suspensions of Lettre cells ( $6 \times 10^7$  cells  $\text{ml}^{-1}$ ), which is the standard for this type of experiment (Bashford, 1994), were incubated with [ $^3\text{H}$ ]5FU (6  $\mu\text{Ci}$ ), [ $^{14}\text{C}$ ]DMO (2  $\mu\text{Ci}$ ) and 0.1 mM or 0.5 mM cold 5FU, in duplicate, in each of a range of McIlwain buffer solutions (from pH 5.0 to 8.0), the final concentration of the buffer being 20 mM (Dawson et al, 1969) for 5, 10 and 20 min. The final volume was 1 ml made up with isotonic saline solution. In some experiments the incubation medium was modified by the addition of 10 mM glucose, or the replacement of  $\text{K}^+$  for  $\text{Na}^+$ . The  $\text{pH}_c$  measurements were checked on a pH meter immediately before the experiment. After incubation, 0.3 ml of cell suspension was layered over 0.1 ml of oil and centrifuged for 10 s revealing

three layers: pelleted cells, an oil layer and the incubation medium (for details see Bashford et al, 1983). A sample of supernatant (60  $\mu\text{l}$ ) was counted in a scintillation counter. The oil (middle layer) was aspirated, and the pellet (bottom layer) was resuspended in 1 ml of lithium nitrate (0.1 M lithium nitrate, 0.003% Triton X-100,  $\text{Na}^+$  and  $\text{K}^+$  free) and dispersed by sonication. An aliquot (200  $\mu\text{l}$ ) of the pellet suspension was counted in a liquid scintillation counter.  $\text{Na}^+$  and  $\text{K}^+$  ions were measured on 0.1 ml of the pellet suspension using lithium nitrate as carrier, on the assumption that total  $\text{Na}^+$  plus  $\text{K}^+$  in the pellet could be used as an indicator of pellet water.

### Calculation of intracellular pH from DMO distribution

The  $\text{pH}_i$  was calculated from the equation (Bashford 1994):

$$\text{pH}_i = \log_{10} \{ D_{\text{int}}/D_{\text{ext}} (10^{\text{pK}^{\text{DMO}}} + 10^{\text{pH}_c}) - 10^{\text{pK}^{\text{DMO}}} \}$$

where  $D_{\text{int}}$  = (pellet c.p.m.  $\times F$ ) / [ $\text{Na}^+$  +  $\text{K}^+$ ];  $F$  = molarity of  $\text{Na}^+$  plus  $\text{K}^+$  in cell water and dilution factor;  $D_{\text{ext}}$  = supernatant c.p.m./aliquot size in  $\mu\text{l}$ ;  $D_{\text{int}}/D_{\text{ext}}$  = ratio of DMO concentration inside/outside the cells; and  $\text{pK}^{\text{DMO}} = 6.3$

The pH gradient ( $\Delta\text{pH}$ ) =  $\text{pH}_c - \text{pH}_i$

### Experiments using carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP)

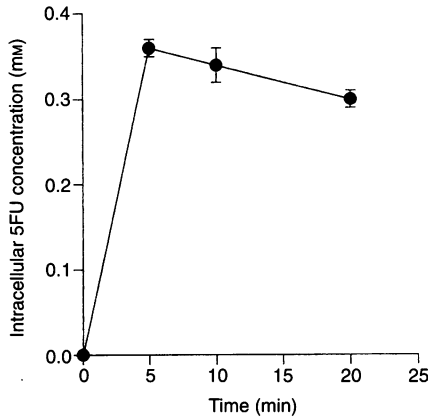
FCCP is an ionophore, which depolarizes plasma membranes making them permeable to  $\text{H}^+$ . Experiments were set up as described above, except for the addition of FCCP (final concentration 10  $\mu\text{M}$ ) or the ethanol vehicle (0.1%) to triplicate samples at  $\text{pH}_c$  of 5, 6, 7 and 8. In half of the experiments, triplicate samples  $\pm$  FCCP were also incubated for 5 min, before centrifugation (10 s), removal of the supernatant, and cold perchloric acid (4%) extraction of the pellet for ATP spectrophotometric analysis as described in Bergmeyer (1974).

### HT29 cells

The human HT29 adenocarcinoma cells were grown in McCoy's medium containing 10% fetal calf serum (FCS) in a 5% carbon dioxide atmosphere at 37°C. After trypsinization using a salt solution of 5% trypsin and 2% EDTA and washing in an isotonic salt solution, cells were resuspended in the McIlwain buffers described above and were used for experiments at a concentration of  $10^6$  cells  $\text{ml}^{-1}$ . All experiments were conducted at room temperature.

### Statistics

Analysis (5FU $_{\text{int}}$ /5FU $_{\text{ext}}$  ratio vs  $\text{pH}_c$ ,  $\text{pH}_i$ , or  $\Delta\text{pH}$ ) involved the correlation coefficient ( $r$ ) significance test where the correlation between the two measurements is significant at the 5% level.  $P$  = probability,  $n$  = sample size and the degrees of freedom =  $n - 2$ . A further assessment of the differences in the degree of correlation of these three parameters with the 5FU $_{\text{int}}$ /5FU $_{\text{ext}}$  ratio was made using an analysis of variance (ANOVA), which is the recommended test when comparing differences between three or more values. Differences in the 5FU $_{\text{int}}$ /5FU $_{\text{ext}}$  ratio  $\pm$  FCCP were tested using a paired  $t$ -test.



**Figure 1** Accumulation of radiolabelled 5FU in Lettre cell pellets. Results are from a single representative experiment and show the calculated intracellular concentration of 5FU determined from triplicate acid-extract samples after separation from the medium after 5, 10 or 20 min incubation with 0.1 mM 5FU at  $\text{pH}_e$  5.8

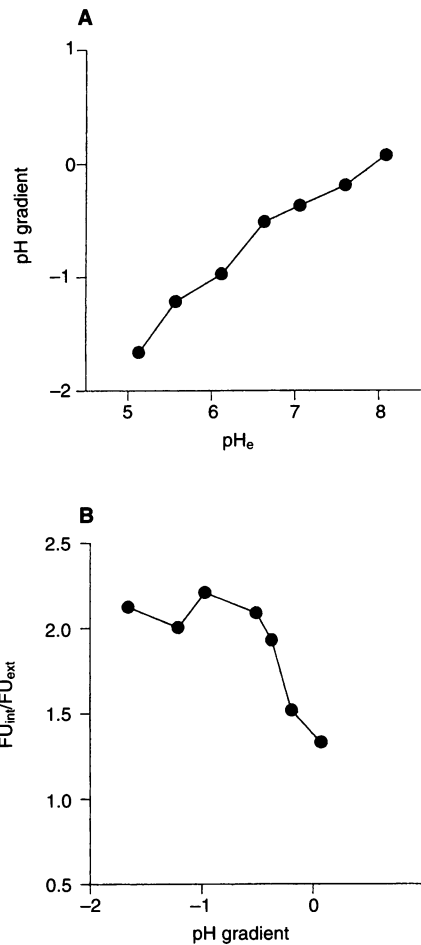
## RESULTS

### Dependence of 5FU distribution on pH

The time-dependent uptake by isolated Lettre cells incubated at room temperature with radiolabelled 5FU is shown in Figure 1. It demonstrates (a) the accumulation of intracellular 5FU (approximately 0.3 mM) and (b) that 5FU equilibrated across the cell membrane within 5 min, as longer incubations of up to 20 min did not significantly increase the amount of radioactivity in the pellet. This suggested that little or no metabolism of 5FU occurred in this time interval. Indeed, Yamamoto and Kawasaki (1981), showed that 5FU metabolism was negligible in Ehrlich ascites cells even after 10 min incubation in medium containing glucose at 37°C, and we have also shown that incubation of Lettre cells with 2 mM 5FU at 37°C for 90 min resulted in only c. 5% 5FU metabolism (McSheehy et al, 1991). Thus, 5FU metabolism in Lettre cells is slow, even in the presence of nutrients, and therefore the distribution of 5FU across the cell membrane after 5 min incubation represented essentially a steady state.

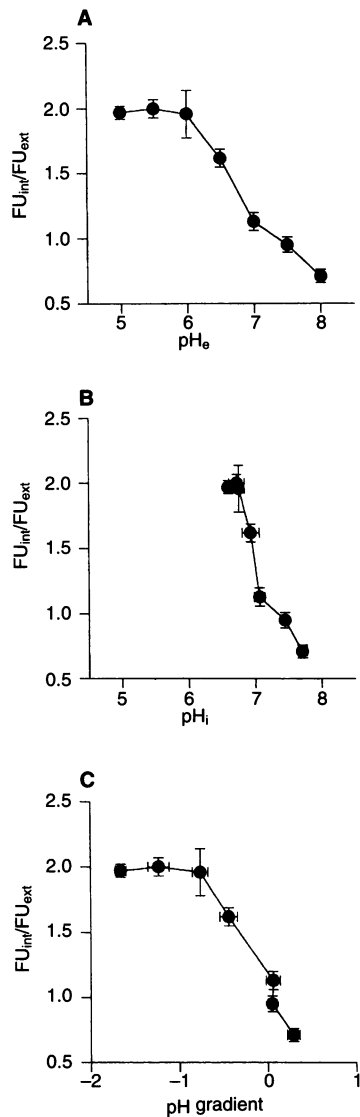
Similar to the radiolabelled 5FU distribution, the incubation time (5, 10 or 20 min) made no difference to [ $^{14}\text{C}$ ]DMO distribution in either the pellet or the supernatant fraction (results not shown). This suggested that equilibration of [ $^{14}\text{C}$ ]DMO along the pH gradient was also reached within the 5-min incubation period, and all subsequent experiments were performed at 5 min. Note that a negative  $\Delta\text{pH}$  ( $-\Delta\text{pH}$ ) demonstrates that the  $\text{pH}_e$  was more acid than the  $\text{pH}_i$ .

Figure 2 shows the relationship between  $\text{pH}_e$  and the  $\Delta\text{pH}$  in one experiment, and how the distribution of [ $^3\text{H}$ ]5FU across the plasma membrane, expressed as the  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  ratio, relates to the  $\Delta\text{pH}$ . The results in Figure 2A show that the more acid the extracellular pH, the larger the  $-\Delta\text{pH}$  was across the cell membranes, i.e. the greater the difference from zero. Over the pH range of 5–8 there was a significant correlation ( $P < 0.001$ ) and this was also unaffected by the incubation time (results not shown). The results in Figure 2B suggested that when the  $\Delta\text{pH}$  became more negative (i.e. the  $-\Delta\text{pH}$  increased), there was a trend towards a higher  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  ratio. The maximum  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  ratio in this experiment, approximately 2:1, was observed at a  $\Delta\text{pH}$  gradient of  $-1$ . At more negative values of  $\Delta\text{pH}$ , there was no further increase in the  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  ratio.



**Figure 2** Relationship between  $\Delta\text{pH}$  across Lettre cell plasma membranes to (A)  $\text{pH}_e$  and (B)  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$ . Results show a single representative experiment performed in duplicate after incubation of cells at  $5 \times 10^7$  cells/ml for 5 min with 0.1 mM 5FU, where  $\text{pH gradient } (\Delta\text{pH}) = \text{pH}_e - \text{pH}_i$

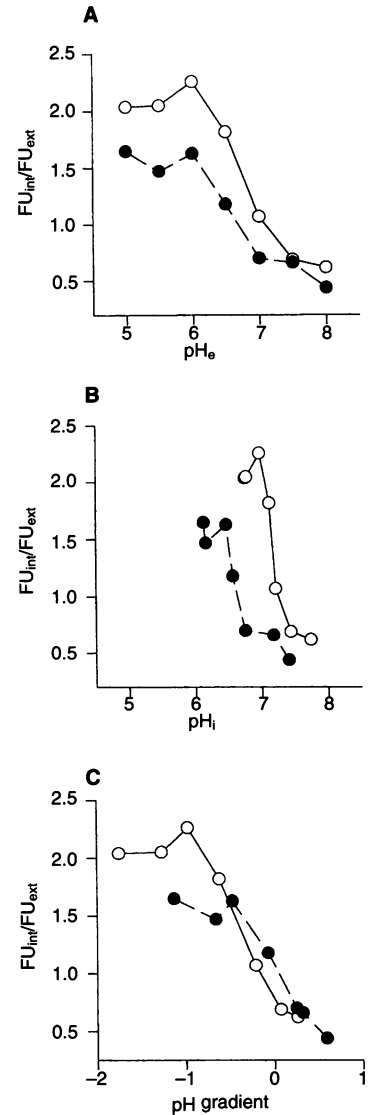
The mean data from several experiments in which the  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  ratio was compared with  $\text{pH}_e$ ,  $\text{pH}_i$  and the  $\Delta\text{pH}$  at an extracellular 5FU concentration of 0.5 mM are shown in Figure 3. A point of inflection occurs in the graph of  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  vs  $\text{pH}_e$  at or above a  $\text{pH}_e$  of 6.0 (Figure 3A). From  $\text{pH}_e$  6–8 there is a highly significant negative linear correlation ( $P = 0.002$ ) with the  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  ratio (i.e. the ratio rises as  $\text{pH}_e$  falls), but no further increase in  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  occurs below  $\text{pH}_e$  6.0. This results in a threefold decrease in the  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  ratio from  $\text{pH}_e$  6 to 8. When the  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  ratio was plotted against  $\text{pH}_i$ , a similar inflection was observed at c.  $\text{pH}_i$  6.8 (Figure 3B), and c.  $-0.8$  for the graph of  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  vs  $\Delta\text{pH}$  (Figure 3C). Again, the linear parts of the graphs in Figure 3B and C showed significant correlation coefficients ( $P = 0.017$  and  $P < 0.001$  for  $\text{pH}_i$  and  $\Delta\text{pH}$  respectively). Similar correlations were seen at an extracellular 5FU concentration of 0.1 mM (results not shown). At 0.5 mM 5FU, the mean  $\pm$  s.d. correlation coefficients in these experiments for the plot of  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  vs pH were  $0.96 \pm 0.03$ ,  $0.98 \pm 0.04$  and  $0.88 \pm 0.03$  for  $\text{pH}_e$ ,  $\Delta\text{pH}$  and  $\text{pH}_i$ , respectively, and an ANOVA demonstrated that  $\text{pH}_e$  and  $\Delta\text{pH}$  were significantly better correlated than  $\text{pH}_i$  with the  $5\text{FU}_{\text{int}}/5\text{FU}_{\text{ext}}$  ratio ( $P = 0.0003$ ). It should be noted that  $\text{pH}_i$  is not an independent variable as the measurement is dependent on the  $\text{pH}_e$  value (see equation in Materials and methods).



**Figure 3** Dependence of the 5FU gradient ( $5FU_{in}/5FU_{ext}$ ) on  $pH_e$ ,  $pH_i$  and  $\Delta pH$  in Lettre cells in the presence of 0.5 mM 5FU. Results show the mean  $\pm$  s.e.m. of 4–6 experiments, where pH gradient ( $\Delta pH$ ) =  $pH_e - pH_i$ . The parameters are significantly correlated over the  $pH_e$  range of 6–8; *P*-values are 0.002, 0.017 and 0.001 for  $pH_e$ ,  $pH_i$  and  $\Delta pH$  respectively

#### Alteration of constituents of the incubation medium

Two experiments were performed in which glucose (10 mM) was included in the incubation medium to cause intracellular acidification from glycolytically produced lactic acid. The 5FU concentration in the extracellular medium was 0.5 mM. Glucose decreased the uptake of DMO: the  $DMO_{in}/DMO_{ext}$  ratio was about 2 with glucose and 3.5 without glucose, implying that intracellular acidification had taken place after glucose was added to the medium, since from the equation on page 6  $pH_i$  is proportional to  $DMO_{in}/DMO_{ext}$ . A representative example of the 5FU results is shown in Figure 4, in which the  $5FU_{in}/5FU_{ext}$  ratio is plotted against  $pH_e$  and  $pH_i$ . The  $5FU_{in}/5FU_{ext}$  ratio did not rise to such a high plateau at acidic  $pH_e$ ; the highest values in the two experiments were 1.6–1.7. Nevertheless, there was still an approximately linear fall in the  $5FU_{in}/5FU_{ext}$  ratio over the pH range 6–8 (Figure 4A), whereas the



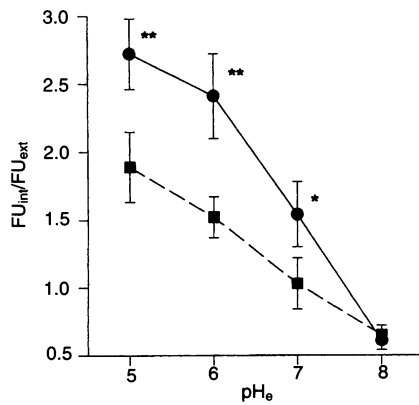
**Figure 4** Effect of glucose on the  $5FU_{in}/5FU_{ext}$  ratio in Lettre cells and correlation with (A)  $pH_e$ , (B)  $pH_i$  and (C)  $\Delta pH$ . Results show a representative experiment performed in duplicate using 0.5 mM 5FU in the presence (●) and absence (○) of 10 mM glucose, where pH gradient ( $\Delta pH$ ) =  $pH_e - pH_i$

$pH_i$  values were clearly shifted to lower values in the presence of glucose (Figure 4B) resulting in a decrease of the  $-\Delta pH$  (Figure 4C).

The importance of the  $Na^+$  gradient in this process was investigated by replacing  $Na^+$  in the incubation medium with  $K^+$ . When the cells were incubated in the presence of 0.5 mM 5FU, there was no decrease in the maximum  $5FU_{in}/5FU_{ext}$  ratio, nor in the pH gradient when two parallel experiments were compared in which  $Na^+$  was present in the medium (results not shown).

#### Effect of FCCP

FCCP depolarizes the plasma membrane making it permeable to  $H^+$  and uncouples oxidative phosphorylation. The effect on 5FU uptake over the  $pH_e$  range 5–8 is shown in Figure 5. The addition of 10  $\mu M$  FCCP during the 5-min incubation reduced by 35% the  $5FU_{in}/5FU_{ext}$  ratio at pH 5, 6 and 7. In addition, there was a small



**Figure 5** Effect of FCCP on the  $5FU_{int}/5FU_{ext}$  ratio in Lettre cells. Results show the mean  $\pm$  sem of eight experiments using 0.5 mM 5FU over the  $pH_e$  range of 5–8 where  $\bullet$  is control and  $\blacksquare$  10  $\mu$ M FCCP. \*\* $P < 0.01$ , \* $P < 0.05$  compared with FCCP-treated (paired *t*-test)

but significant decrease in the  $pH_i$  of 0.12 at  $pH_e$  6 and 7, while both the intra- and extracellular  $[Na^+]$  and  $[K^+]$  were unchanged by FCCP treatment at all  $pH_e$  values. In four of these eight experiments, the total intracellular [ATP] was determined to be  $1.0 \pm 0.1 \mu\text{mol g}^{-1}$  (mean  $\pm$  sem), and FCCP caused a mean decrease in this concentration of 33%.

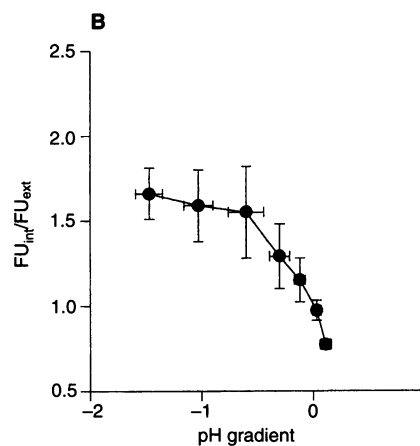
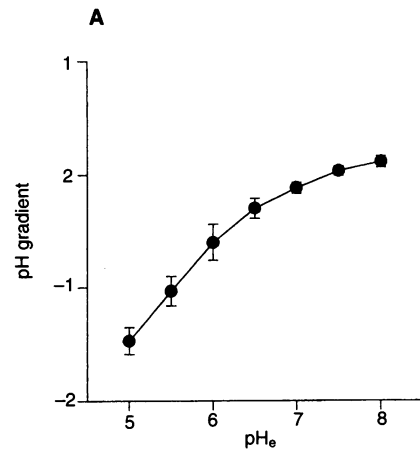
#### Distribution of 5FU across HT29 colon adenocarcinoma plasma cell membranes

Similar results to those found in the Lettre cells were also found when human HT29 cells were incubated with 5FU (Figure 6). Using the same  $pH_e$  range of 5–8, the maximum recorded  $\Delta pH$  was  $-1.5$  (at  $pH_e$  5), resulting in a mean  $5FU_{int}/5FU_{ext}$  ratio of 1.66. This ratio was significantly different ( $P < 0.05$ ) from that measured when the  $\Delta pH$  was close to zero ( $pH_e$  7 or 7.5). When the  $5FU_{int}/5FU_{ext}$  ratio was plotted against  $pH_e$ , there was a highly significant negative correlation over the  $pH_e$  range 6–8 ( $P = 0.0003$ ), similar to that found for Lettre cells.

#### DISCUSSION

We have studied the pH dependence of 5FU uptake in two tumour cell lines: the human HT29, which has been used in  $^{19}F$ -MRS pharmacokinetic studies *in vivo* (McSheehy et al, 1997), and murine Lettre cells in which many plasma membrane studies have been performed (Bashford and Pasternak, 1984). Variation of the pH of the suspending medium ( $pH_e$ ) was used to alter  $\Delta pH$ , i.e. the difference between  $pH_i$  and  $pH_e$ , so that a  $-\Delta pH$  of 1.5–2.0 could be attained at  $pH_e$  5. An increase in the  $-\Delta pH$  correlated with an increase in the  $5FU_{int}/5FU_{ext}$  ratio, i.e. uptake of 5FU by the cells. This 5FU uptake by Lettre cells was markedly inhibited in the presence of the ionophore FCCP.

How can the relationship between 5FU uptake and pH be explained? In the absence of significant metabolism, the most obvious explanation would be that the drug is distributed across the cell membrane as a weak acid. The enolic hydroxyl groups of 5FU ionize as acids, but with a  $pK_a$  of 8.1. If 5FU distributed like a weak acid, little intracellular accumulation would be expected under physiological conditions, because  $pK_{5FU} > pH_i$ . However, in



**Figure 6** Relationship between  $\Delta pH$  across HT29 cell plasma membranes and (A)  $pH_e$  and  $5FU_{int}/5FU_{ext}$ . Results show the mean  $\pm$  sem from four experiments incubating  $10^6$  cells  $\text{ml}^{-1}$  for 5 min with 0.1 mM 5FU, where  $pH$  gradient ( $\Delta pH$ ) =  $pH_e - pH_i$

the presence of 0.5 mM 5FU at a  $pH_e$  of 6.0 and  $pH_i$  of 6.7 (where the  $5FU_{int}/5FU_{ext}$  of  $\geq 2:1$  was measured in the Lettre cells – see Figure 3), the calculated ratio would be about 1.0:1. At  $pH_i$  7.31 and  $pH_e$  6.63, which are the pH values more typical of physiological conditions, the calculated ratio would still only be 1.15:1. Thus, our observations cannot be explained simply on the basis that 5FU distributes as a weak acid.

Wohlhueter et al, (1980) showed that transport of 5FU into Novikoff rat hepatoma cells was inhibited by uracil, suggesting that 5FU uptake involved facilitated transport via the uracil transporter. They also showed that the initial rate of transport was dependent on pH and related to  $pK_{5FU}$  as only the uncharged species is transported. The  $pK$  of uracil is 9.2, so it will be almost entirely in the uncharged form at all reasonable physiological pH values. 5FU, despite having a lower  $pK$  of 8.1, would be 90–95% in the uncharged form at around  $pH_e$  6.5–7.0 (the most likely  $pH_e$  values to be encountered in tumours), whereas at a  $pH_e$  of 6.0, 99% would be uncharged. In both cases, near-maximum initial rates of transport will be allowed, whereas at  $pH_e$  8.0, 50% of the 5FU would be charged and the initial rate of transport would be markedly reduced. These considerations do not explain the 'steady-state' 5FU distribution observed in the present experiments. This is because the

uracil transporter will transport the uncharged form of its 'ligand' equally efficiently either into or out of the cell, the amount of 5FU transported in either direction depending on the concentration of uncharged 5FU available on the inside and outside of the cell membrane. In our experiments, 5FU retention only occurs at pH values (intracellular or extracellular) in the range of 6–7, when the concentration of the uncharged species would not be significantly different from one side of the plasma membrane to the other. In addition, Wolhueter's work investigated initial rates of 5FU transport, whereas our experiments study an equilibrium, or steady state, that is unchanged for up to 20 min after incubation (Figure 1).

Yamamoto and Kawasaki (1981) studied facilitative transport in Ehrlich ascites cells (the cell line from which Lettre cells were derived) suspended in 5 mM glucose, and showed that 5FU uptake was directly coupled to the ATP derived from glycolysis. This active transport occurred in the absence of a Na<sup>+</sup> gradient. The initial rate was slightly stimulated by Na<sup>+</sup> (1.3-fold), suggesting that Na<sup>+</sup> here was acting as a co-factor and not in creating an electrochemical potential, as occurs in the active transport of glucose and many amino acids. They concluded that 5FU uptake in these cells resembled the ATP-driven, Na<sup>+</sup>-independent amino acid transport that occurs in *Escherichia coli*. We also found that the 5FU<sub>int</sub>/5FU<sub>ext</sub> ratio was unaffected by the absence of Na<sup>+</sup> in the incubation medium, suggesting that in the steady state it was Na<sup>+</sup> independent.

An alternative explanation is that the electrochemical concentration gradient of H<sup>+</sup> that we have imposed across the plasma membrane, provides a proton motive force (p.m.f.) which can be used to perform transport work. The p.m.f. (or Δp) is related to the ΔpH:

$$\Delta p = \Delta \Psi - (Z\Delta pH)$$

where Z is a constant of 59 mV at 37°C and ΔΨ is the plasma membrane potential (Harrison and Lunt, 1980). Co-transport of 5FU may occur by proton symport, as long as sufficient p.m.f. exists, i.e. there continues to be a -ΔpH or the ΔΨ is not depolarized. This hypothesis predicts that there would be no intracellular accumulation of 5FU in the absence of a -ΔpH across the cell membrane, or when the plasma membrane is depolarized. Figure 3C (Lettre cells) and Figure 6B (HT29 cells) show that the 5FU<sub>int</sub>/5FU<sub>ext</sub> ratio is indeed found to be 1.0 when the ΔpH across the cell membrane is zero, and, similarly, the 5FU<sub>int</sub>/5FU<sub>ext</sub> ratio is <1.0 when the cell membrane ΔpH becomes positive (i.e. pH<sub>c</sub> is more alkaline than pH<sub>i</sub>). Table 1 shows the calculated p.m.f. for Lettre cells over the pH<sub>c</sub> range studied. Note that p.m.f. decreases by approximately two-fold from pH<sub>c</sub> 5 to pH<sub>c</sub> 8, which is a similar change in magnitude to that measured for the 5FU<sub>int</sub>/5FU<sub>ext</sub> ratio.

The hypothesis was tested experimentally by incubating Lettre cells with FCCP. FCCP is an ionophore which depolarizes plasma membranes, making them permeable to H<sup>+</sup>, and uncouples mitochondrial oxidative phosphorylation. Under the conditions of our study, FCCP significantly decreased the 5FU<sub>int</sub>/5FU<sub>ext</sub> ratio by 35% at pH<sub>c</sub> 5–7, and the total intracellular [ATP] by a similar amount. As the ion gradients were unchanged (as assessed by the distribution of K<sup>+</sup> and Na<sup>+</sup> across the plasma cell membrane), we have hypothesized that the [ATP] was still sufficient to maintain ion gradients, and that the depolarization of the plasma membrane was responsible for decreasing the 5FU<sub>int</sub>/5FU<sub>ext</sub> ratio. This is consistent with the idea that 5FU uptake is dependent on p.m.f. However, these experiments are in no way conclusive evidence of our hypothesis, and further investigations are being pursued to distinguish between the depolarizing and uncoupling effects of FCCP.

**Table 1** Changes in ΔΨ and p.m.f. in Lettre cells as a function of ΔpH

pH <sub>c</sub>	ΔpH	ΔΨ (mV)	p.m.f.	5FU <sub>int</sub> /5FU <sub>ext</sub> ratio
5	-1.66	-4	99	1.97
5.5	-1.23	-15.5	86	2
6	-0.76	-27	70	1.96
6.5	-0.44	-38.5	64	1.62
7	0.06	-50	47	1.13
7.5	0.05	-61.5	59	0.95
8	0.29	-73	57	0.71

Values for pH<sub>c</sub>, ΔpH and the FU ratio (FU<sub>int</sub>/FU<sub>ext</sub>) are taken from the values in Figure 3 (mean of five experiments), while ΔΨ is calculated from the data in Bashford and Pasternak (1984) in which for these cells, the ΔΨ depolarizes by 23 mV for each unit decrease in the pH<sub>c</sub>, when ΔΨ = -57 at pH 7.3. p.m.f. (Δp) is calculated from the equation: Δp = ΔΨ - ZΔpH, in which Z is a combination of constants equal to 59 mV.

Our results and hypothesis are consistent with the MRS observations of Guerquin-Kern et al (1991), who showed that more 5FU was retained in a rat fibrosarcoma at lower pH<sub>i</sub> (< 6.9), induced by glucose infusion, than at pH<sub>i</sub> 7.3, similar to the findings made in this study. Furthermore, Gerweck et al (1991) found that in tumours administration of glucose to the host induced a decrease in pH<sub>i</sub> but caused an even greater decrease in pH<sub>c</sub> 60 min later, thus increasing the -ΔpH still further. Thus, although pH<sub>c</sub> was not measured in the study of Guerquin-Kern et al (1991), it is possible that the increased retention of 5FU was due to an increased -ΔpH across the tumour cell membranes. The results of our experiments in which glucose was added to the medium, reported in Figure 4, are broadly in agreement with our hypothesis. Glucose lowered the pH<sub>i</sub>, and thus decreased the -ΔpH (Figure 4C) and consequently the 5FU<sub>int</sub>/5FU<sub>ext</sub> ratio.

Prerequisite for 5FU cytotoxicity is intracellular metabolism of the prodrug to 5-fluoronucleotides, and thus the first step necessary for anti-tumour activity is 5FU uptake by the target cells. Furthermore, the therapeutic ratio will be enhanced if uptake into normal cells is minimized. Our results suggest ways in which both of these favourable effects occur with 5FU. At a lower pH<sub>c</sub>, in which the -ΔpH is large, such as occurs in solid tumours, there would be an enhancement of 5FU uptake, whereas normal cells (in which ΔpH is usually positive) will tend to exclude 5FU. In solid tumours this could lead to an accumulation of 5FU relative to other normal tissues, which is precisely what has been recorded in biopsies (Peters et al, 1993) and also by non-invasive <sup>19</sup>F MRS in animal models and in the clinic (Findlay et al, 1993; Presant et al, 1994). The hypothesis suggests potential means of manipulating the tumour pH environment for therapeutic gain. For example, one of the many drugs used in combination with 5FU in the clinic is interferon α, which has been shown in vitro to increase activity of the plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter in human tumour cells leading to an increase in pH<sub>i</sub> (Maheshwari et al, 1991). Indeed, we have recently shown in HT29 tumours xenografted in nude mice that interferon α increased the -ΔpH via an increase in pH<sub>i</sub>, and increased 5FU retention in these tumours (McSheehy et al, 1997).

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