Sodium Fluorescein Accumulation in Cultured Cells

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Abstract. The mechanism of intracellular fluorescein accumulation was investigated by studying fluorescein accumulation kinetics in individual cells in culture. The experiments were done with K562 human erythroleukemia cell culture and a primary culture of human embryonic skin fibroblasts. Various experimental conditions were used by varying pH and ion composition of the external media, and/or temperature. All the experimental results obtained are in support of the proposed theoretical model describing fluorescein accumulation in cells along the pH gradient. In addition, we could show that fluorescein molecules are bound to some intracellular macromolecules. Fluorescein accumulation in the cells studied does not depend on their malignant nature. It is therefore reasonable to conclude that fluorescein accumulation observed in clinical investigations of human gastric cancer is governed by the pH gradient between the cells and the extracellular fluid; this gradient is much greater in the tumor than in normal tissues.

Key words: Cancer diagnostic — Dye accumulation — Fluorescein dye — Cells in culture

Introduction

Numerous attempts have been undertaken to find a dye that would meet the requirements of photodynamic therapy and fluorescence diagnosis of malignant tumors. Different dyes accumulate selectively in malignant tissues (Berns 1984; Moan et al. 1987; Cincotta et al. 1987; Balchum et al. 1987; Davis et al. 1985). Selective accumulation of sodium fluorescein (Fl) in malignant tumors was first observed in rabbit brain tumors (Shargorodskii et al. 1955) and later en experimental tumors in mice (Doughery 1974).

We studied Fl accumulation in malignant human gastric tumors. Clinical observations were carried out in different hospitals in Moscow, (Dzbanovski et al.

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1989; Polsacnev and Potemkina 1989; Braginskaja et al. 1993). For clinical examination the stomachs were endoscopically examined under ordinary white light and under blue light that stimulates fluorescein fluorescence. Fluorescein was detected either by visual method or using a special fluorimeter. About 500 patients with gastric diseases were examined. A correct diagnosis of cancer was made in about 85% of cases.

Knowledge of dye penetration mechanism into the cell is very important with respect to dyes used in the fluorescence diagnostics of cancer.

Our first study of Fl penetration into cells (Kalaidzidis et al. 1987) used experiments with cell suspension. Two cell lines were used: normal mouse fibroblasts NIH 3T3 and the same fibroblasts transformed by the oncogen virus (EJ 3T3). Fl accumulation inside EJ 3T3 cells was studied and acidification of the solution surrounding these cells to pH value of about 5.7 was observed. Under the same experimental conditions normal NIH 3T3 cells neither accumulated Fl nor acidified the surrounding media. It was proposed that a change of proton concentration in the medium caused by the glycolytic respiration of the transformed cells may play a role in the intracellular accumulation of the dye.

It has been now established that the extracellular fluid in malignant tumors is more acidic than in the normal tissues (Tannock and Routin 1989; Newell and Tannock 1989). Moreover, recently it has been established that pH inside a tumor cell is neutral or slightly alkaline (Griffiths 1991). The pH values inside tumor cells and normal tissues were all in a range between 6.9 and 7.4, while pH of the extracellular fluid for normal tissues was between 7.2 and 7.6. Microelectrode studies of tumors, on the other hand, gave a much wider range of extracellular pH values, (5.6–7.6) with mean values mainly on the acidic side (Griffiths 1991). It is therefore reasonable to expect that Fl accumulation in tumors *in vivo* is governed by the pH gradient between the cells and the extracellular fluid.

The aim of this paper is to present the results of our investigation of the mechanism of intracellular Fl accumulation. We studied Fl accumulation kinetics in individual cells in culture. The experiments were carried out at various pH values of the external media and various temperatures. The theoretical model suggested that Fl is being accumulated as a function of the pH gradient. The results obtained confirm the theoretical predictions.

Materials and Methods

Cell cultures

The following cell cultures were used: K562 human erythroleukemia cells and a primary culture of human embryonic skin fibroblasts. The cells were cultured in Raso Therm vials in DMEM (Sigma, St. Louis, MO, USA) and RPMI 1640 (Flow Lab, Meckenheim, FRG) media supplemented with 7% calf embryonic serum (Sigma) and 2 mmol/l L-glutamine (Sigma, St. Louis, MO, USA).

Primary culture of skin fibroblasts was obtained from the skin of 12–14 weeks old human embryos. The grafts were washed for 1 h in three volumes of Eagle's medium containing 1 000 units/ml penicillin, 1 mg/ml of streptomycin and 7.5 μg/ml of fungisone; they were then placed in 0.25% trypsin solution for 18 h at 4 °C and transferred into a mixture of 0.25% trypsin solution and 0.02% sodium ethylenediamine tetra-acetate (1:1) where they were disscociated for 30 min under gentle stirring. After inactivation of trypsin by the same volume of bovine embryonic serum, the suspension was filtered through a double gauze layer and centrifuged for 10 min (at $200 \times g$). The sediment was resuspended in a definite amount of the growth medium and counted using a Goryaev chamber. The resuspended cells were inoculated onto cover plates placed in plastic Petri dishes, at a concentration of 10^5 cells per 1 ml of growth medium. The Petri dishes were placed in a CO_2 incubator (ASSAB, Sweden). The medium was changed once every 3 days. The composition of the growth medium was as follows: Eagle's medium (Sigma, St. Louis, MO, USA) and a medium containing 0.5% lactalbumin hydrolyzate in Hanks' (balanced salt) solution (Sigma, St. Louis, MO, USA) (1:2), 1 mmol/l of sodium pyruvate (Sigma, St. Louis, MO, USA), 2 mmol/l of L-glutamine (Sigma, St. Louis, MO, USA) and 10% bovine embryonic serum (Sigma, St. Louis, MO, USA). Cellular measurements were carried out 3–4 days after the inoculation of the primary culture.

One day before the experiment, the K562 cells were inoculated onto cover glasses placed in Petri dishes and transferred into a CO_2 incubator. Even though the K562 cells are grown as a suspension they readily adhere to the substrate preserving their spheroid shape.

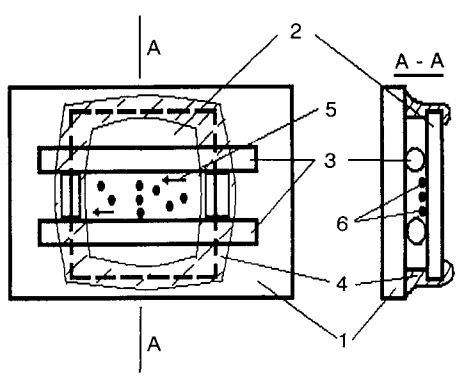

Figure 1. A schematic illustration of the perfusion chamber. 1 – object glass; 2 – cover glass; 3 – glass fibers; 4 – paraffin; 5 – Fl solution flow; 6 – K562 cell.

For each experiment, a special perfusion chamber was used. It is schematically illustrated in Fig. 1. The chamber was constructed in the following way. A cover glass (2) with the adhered cells was placed on an object glass (1). The height of the solution layer between the bottom glass and the cover glass was maintained by the introduction of 25 μm glass fibers (3). The enclosed culture chamber was thoroughly sealed with paraffin (4), two holes being left for the flow (5). The chamber volume was 1.5–2 microliters. The rate of solution exchange was approximately 0.5 ml/l. The construction of the chamber

allows to investigate separate cells (6). To control and to provide a constant temperature of the experimental sample a special thermostatic holder was used for the observation of fluorescence under a microscope.

The experiments were conducted in balanced Dulbecco's saline buffer (pH values between 6.2 and 6.8). The pH value was varied by changing the ratio of Na_2HPO_4 to KH_2PO_4, and the exact value was checked by Beckman Model 4500 digital pH meter with an accuracy of 0.03 pH units. In a special series of experiments the Dulbecco's saline buffer was used without Ca^{2+} or Mg^{2+} ions.

The sodium salt of fluorescein (Sigma, St. Louis, MO, USA) used was not previously purified. Its concentration in buffer solutions for cell incubation was 2×10^{-5} mol/l.

Microscopic intracellular fluorescence measurements

Laser (He-Cd laser LPM-11, $\lambda = 442$ nm) beam was directed through a luminescence microscope (objective ×25) to excite fluorescence of the entire microscopic field (0.5 mm× 0.5 mm), at an intensity of 50 mW/cm.

The image of the fluorescent cells was recorded using a sensitive BM981A SIT camera (BM Spectronik, FRG). The camera image was digitized.

During the investigations of the Fl accumulation kinetics the fluorescence image was measured every 5 minutes after the addition of the dye. The time necessary to measure the fluorescence signal was a few seconds. The cells were exposed to the exciting laser light for this short period only. The photobleaching was less than 1% in this case. Autofluorescence of the cells was negligibly small compared to Fl fluorescence, and could not be detected.

The digitized fluorescence image was processed using an E3 computer (ELTEC, FRG). The data showed that Fl was present mainly in the cytoplasm. The cell image was about 20 pixels in diameter. To obtain cell fluorescence we averaged the signal for 10×10 pixel squares for each cell. The calculations were done for each cell in the microscopic field. Thus the fluorescence signals inside and outside the cell were measured. The difference between the cell diameter and the height of the Fl solution was taken into account and the true fluorescence of the cell was determined. Then the ratio of fluorescence inside and outside the cell (F) was calculated. The experimental error for F determination was 0.03.

The principal parameter characterizing the process of Fl penetration into the cell is the coefficient of accumulation (N), i.e., the ratio of Fl concentration inside the cell, C_i, to the outside concentration of the dye, C_0: $N = C_i/C_0$. The two parameters F and N may differ because of the possible difference between Fl fluorescence quantum yield inside the cell and that in the external media. It should be mentioned that it is parameter F that is important for fluorescent diagnostics of malignant cells.

In a special series of experiments the intracellular pH value was determined.

Measurements of intracellular pH in a cell were based on the fact that the shape of the Fl fluorescence spectrum depends on the pH value of the solution; therefore, Fl and its derivatives are widely used as fluorescent pH indicator (Visser et al. 1979; Thomas et al. 1979; Rotenberg et al. 1983; Geisow 1984; Bidet et al. 1988).

We chose the ratio of fluorescence intensities at wavelengths of $\lambda_1 = 518$ nm and $\lambda_2 = 550$ nm as a parameter characterizing the shape of the Fl fluorescence spectrum (that made it possible to determine the pH). In this case the laser beam was focused by the microscope lens to 5–7 μm. Instead of a SIT camera the cassette with two interference filters at wavelengths λ_1 and λ_2 and then the photomultiplier were put on the top of the microscope. Thus the fluorescence intensities at λ_1 and λ_2 were detected and the ratio

$I(\lambda_1)/I(\lambda_2)$ could be determined. The calibration curve for the ratio vs. pH value was recorded using Nigericin (Thomas et al. 1979). Using the experimental ratio $I(\lambda_1)/I(\lambda_2)$ for the cells investigated and the calibration curve we could determine the pH value inside the cell. The error of the pH value determination was less than 0.05 units.

Results and Discussion

The theoretical model of Fl accumulation in cells

We have shown (Kalaidzidis et al. 1987) that upon acidification of the external medium the cells in culture started accumulating Fl. The pH gradient was proposed to play a dominant role in the intracellular accumulation of the dye because of the more alkaline pH inside the cell.

One could propose a simple model for the penetration of the weak acid sodium fluorescein salt into the cells according to the pH gradient. It is known that Fl occurs as dianion, monoanion or a neutral molecule. The mutual ratio of the three forms depends on the pH value of the medium. The constant of the monoanion-dianion equilibrium is $K_1 = 10^{-6.7}$ mol/l, and that of the monoanion-neutral molecule equilibrium is $K_2 = 10^{-4.4}$ mol/l (Shah et al. 1983). Fl accumulation in more alkaline pH media (i.e. inside the cell) may proceed provided that the neutral Fl molecule is readily dissolved in the lipid membrane and can passively diffuse into the cell. After permeation into the cell (with more alkaline pH values), a different equilibrium is established with the proportion of the neutral form decreases, and that of the dianion increase. The newly formed anions cannot escape the cell through the membrane. The situation is similar to that for HpD (Pottier and Kennedy 1990). In order to account for Fl binding with macromolecules inside the cell, we assume that intracellular binding proceeds much faster than Fl penetration. Then the concentration of Fl bound inside the cell (C_i^b) is:

$$C_i^b = S\,C_i^f, \tag{1}$$

where S is the parameter characterizing the Fl binding with different macromolecules inside the cell, and C_i^f is the concentration of free Fl molecules in the cell. Let $C_i = C_i^f + C_i^b$, be a total Fl concentration inside the cell; then

$$C_i^f = C_i/(S+1) \tag{2}$$

Thus, temporary changes in Fl concentration inside the cell (C_i) depend on two fluxes. First, on the flow of neutral Fl forms from the external medium to the cell $C_0\alpha_0 k$, where C_0 is the Fl concentration in the external medium, α_0 is the fraction of neutral molecules in the external medium, and k is the parameter of the membrane permeating properties. The second factor is the flow of the neutral

portion of free Fl molecules from the cell to the external medium $C_i\alpha_i k/(S+1)$, where α_i is the fraction of neutral Fl molecules inside the cell. Thus,

$$dC_i/dt = C_0\alpha_0 k - C_i\alpha_i k/(S+1) \tag{3}$$

The Fl concentration inside the cell is time-dependent:

$$C_i(t) = C_0(S+1)(\alpha_0/\alpha_1)\cdot(1-\exp(-t/\tau)), \tag{4}$$

where

$$\tau = (S+1)/k\alpha_i \tag{5}$$

The total coefficient of accumulation is:

$$\begin{aligned} N(t) &= C_i(t)/C_0(t) = N_m(1-\exp(-t/\tau)), \\ N_m &= (S+1)\alpha_0/\alpha_i, \qquad \tau = (S+1)/k\alpha_i \end{aligned} \tag{6}$$

For the parameter $F(t)$ we can write the expression:

$$F(t) = F_m(1-\exp(-t/\tau)),\ \tau = (1+S^*)/K\alpha_i)$$

where (7)

$$F_m = (S^*+1)(\Phi_i^f/\Phi_0)(\alpha_0/\alpha_i)$$

is the theoretical maximum value F that can be attained during accumulation, Φ_i^f, Φ_i^b and Φ_0 are the quantum yields of free and bound Fl in the cell and of Fl outside the cell respectively. $S^* = S\Phi_i^b/\Phi_i^f$ is the binding parameter accounting for the difference between Φ_i^f and Φ_i^b.

The fraction of neutral Fl molecules α is rigidly imposed by the pH value:

$$\alpha = [H^+]^2/([H^+]^2 + K_2[H^+] + K_1K_2), \tag{8}$$

Where $[H^+]$ is the concentration of hydrogen ions.

It should be noted that under our experimental conditions (extracellular pH ranging between 6.2–6.8 and the intracellular pH value ranging between 6.6–7.0) the value of Φ_i^f for Fl is close to the value of Φ_0. The value of Φ_i^b is known to be about 0.43 Φ_0 (Sernetz and Thaer 1972). Therefore S* is about 0.43 S and $F_m = (1+S^*)\alpha_0/\alpha_i$.

In vitro cell Fl accumulation kinetics; a comparison experimental values and theoretical expectations

It is evident that more detailed information about the mechanism of the dye penetration into the cell can be obtained by studying the kinetics of the accumulation

process. We believe that the perfusion chamber described above provides new opportunities for this purpose.

By using the chamber we could observe the cells just after the staining and record the whole process in real time. One and the same sample, and even one and the same cell, can be used to observe Fl redistribution following a change in the external conditions. And finally, the chamber allows maintaining of external conditions (pH value, oxygen concentration, etc.) for the cells in the chamber constant throughout the experimental procedure.

Fig. 2 shows examples of experimental kinetics of Fl accumulation for two cells of K562 human erythroleukemia.

According to our theoretical model the kinetics of accumulation is described by eq. 7. In this case, it is convenient to characterize the kinetics by two parameters: maximum value F_m, and τ, the latter describing the rate of the process. These parameters were obtained by approximation of the experimental data using eq. 7 and the least square root method. The values for F_m, τ and χ^2 are shown in Fig. 2. The theoretical curves are also presented.

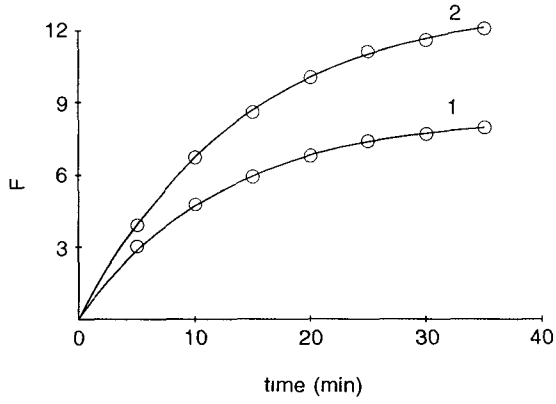

Figure 2. The kinetics of Fl accumulation, $F(t)$, in an isolated K562 cell. 1 – $F_m = 8.5$; $\tau = 12$ min; $\chi^2_5 = 1.3$; reliability = 94%; 2 – $F_m = 13.2$; $\tau = 14$ min; $\chi^2_5 = 2.4$; reliability = 81%.

As can be seen from (7) and (6), the parameters F_m and τ are interrelated: $F_m = (\Phi_i^f/\Phi_0)\, k\alpha_0\tau$. In other words, the higher the value of F_m, the longer the period necessary to obtain it. This conclusion is experimentally proved as illustrated by Fig. 2, where $F_{m2} > F_{m1}$ and $\tau_2 > \tau_1$, respectively.

The considerable heterogeneity of the cell population requires a large body of experimental data. We measured Fl accumulation kinetics for about 200 K562 cells. For each cell a pair of F_m and τ values was obtained by approximation of the experimental kinetics using eq. 7. Different F_m values were observed for a constant τ value. This phenomenon may be due to changes in membrane viscosity, i.e. the magnitude of k. We put the results obtained for 200 K562 cells in increasing order of

τ values. Than mean values and standard deviations for τ and F_m were calculated for every 10 points in a row. The results of these calculations are presented in Fig. 3. The experiments were carried out in Dulbecco's buffer at pH 6.2. It can be seen that, in general, an increase of F_m is actually paralleled by a prolonged period of accumulation τ. This agrees with the theoretical model. The same Figure gives $F_m(\tau)$ data for pH 6.8 in Dulbecco's buffer.

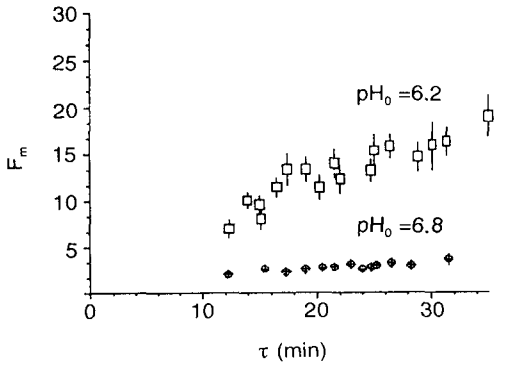

Figure 3. The parameter F_m (see the text) as a function of τ for K562 cells.

Table 1. Mean values and the standard deviations of the parameters F_m and τ (see the text) obtained for various external pH values. Each value was obtained for 400 cells.

External pH	K562 cells		Fibroblasts of human embryonal skin	
	F_m	τ (min)	F_m	τ (min)
6.2	14 ± 4	16 ± 5	13 ± 4	17 ± 5
6.5	7 ± 3	24 ± 7	4.3 ± 1.5	16 ± 4
6.8	2.1 ± 0.6	23 ± 7	–	–

Table 1 gives the mean values of F_m and τ for the three pH values of the external medium studied. It can be seen that the range of changes of τ is quite small while the value of F_m decreases essentially as the pH of external medium increases. The data obtained confirm the results of earlier experiments done with cell suspensions (Kalaidzidis et al. 1987) that suggested a change in proton concentration in a medium to play a role in the intracellular accumulation of the dye.

In a special series of experiments the Dulbecco's saline buffer was used without Ca^{2+} or Mg^{2+} ions. The Fl accumulation kinetics measured did not differ from that observed in the previous experiments.

A special experimental series was designed to study the kinetics of Fl accumulation in fibroblasts of a primary human skin culture. Table 1 shows the mean values of F_m and τ for these measurements. The results obtained for the kinetics of accumulation in the primary skin cultures agree with those obtained for human erythroleukemia cells.

The intracellular pH measured by the method described above varied between 6.6 and 7.0, the average value being 6.8.

The experimentally determined intracellular pH values could be used to calculate the α_0/α_i ratio using formula (8) and to compare it with the experimentally obtained parameter F_m. The coefficient of proportionality $(S^*+1)\,(\Phi_i^f/\Phi_0)$ can be obtained. This coefficient appears to be approximately equal to 3. Hence, $S \neq 0$; it can than be stated that intracellular binding plays a role in Fl accumulation.

We studied the binding phenomenon of Fl molecules in a special series of experiments. In these experiments cells in the perfusion chamber were placed into 70% ethanol as a fixative substance for about 10 minutes. Subsequently, the cells were immersed into Fl solution in the same manner as in the previous experiments without preliminary fixation. The accumulation kinetic was observed and the parameter F_m was determined for each cell in the microscopic field.

The mean value of parameter F_m was 3.0 ± 0.4 for pH 6.8. It is well known that ethanol fixation damages the membrane. Consequently, the medium inside and outside the cell can be expected to be identical. In this case, the parameter F_m would characterize the binding of Fl molecules only. F_m is equal to $(S^*+1)\,(\Phi_i^f/\Phi_0)$ (eq. 7). The experimental value is in good agreement with the previous theoretical estimation.

The temperature dependence of the kinetics of Fl accumulation

The above data concerning the kinetics of Fl accumulation by cells in culture were obtained under a constant temperature of 25 °C, and the results confirmed the theoretical model of passive diffusion of neutral Fl molecule through the cell membrane and Fl accumulation according to the pH-gradient. The investigations of Fl accumulation in the absence of Ca^{2+} and Mg^{2+} ions showed no changes in the F_m and τ values. This made us to conclude that no active transport of Fl molecules through the cell membrane is probable.

In a next series of experiments it was interesting to study the temperature dependence of intracellular Fl accumulation. Cells of human erythroleukemia K562 were investigated.

Fl accumulation was studied in balanced Dulbecco's saline buffer, pH 6.2. The temperature was varied between 13 °C and 37 °C, and was held constant throughout an experiment. As in the previous experiments the dependence $F_m(\tau)$ was determined using data of accumulation kinetics. For a constant temperature the prolongation of the accumulation period τ was accompanied by an increase of

maximum value F_m, as in the previous experiments. The mean values of F_m and accumulation time τ are presented in Table 2 for five temperatures examined.

Table 2. Mean times of accumulation τ, maximum values F_m, and membrane permeabilities k for K562 cells in Dulbecco's buffer, pH 6.2, at different temperatures. Each mean value and standard deviation were obtained for 400 cells.

T (°C)	τ (min)	F_m	k (s^{-1})
13°C	72 ± 13	10.4 ± 1.8	0.23
20°C	46 ± 13	10.7 ± 2.5	0.36
25°C	24 ± 5	14.0 ± 4.0	0.70
28°C	18 ± 6	12.5 ± 4.0	0.93
37°C	14 ± 4	9.0 ± 2.8	1.2

The fact that temperature variations were accompanied by changes in the rate of Fl permeation through the cellular membrane only and that the final accumulation coefficient remained unchanged is in good agreement with the theoretical hypothesis.

It has already been mentioned that special spectral measurements based on Fl fluorescence spectra gave a mean value of cellular pH of about 6.8. This allowed us to determine the value of α_i. Using the results of experiments designed to study the binding process, we could obtain a measure of the cell membrane permeability $k = (S^* + 1)/(\tau \alpha_i)$ (eq. 4, Table 2).

The temperature dependence of membrane permeability k is shown in Fig. 4. As can be seen an abrupt change in cell membrane permeation properties appears at near 20°C.

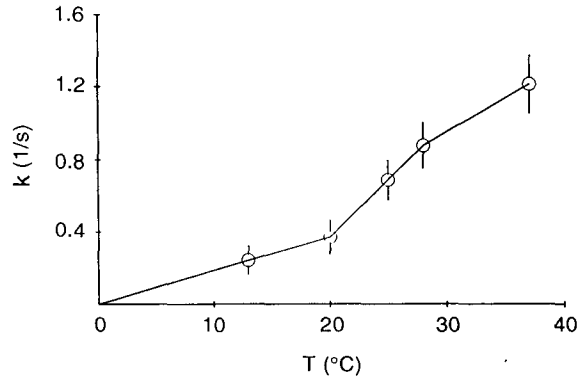

Figure 4. The dependence of the membrane permeability k on temperature T (°C).

All the experimental results obtained support the proposed theoretical model describing Fl accumulation in the cells in dependence on the pH gradient. Moreover, we could show that similarly as with HPD (Chang and Dougherty 1978; Moan et al. 1981), neither Fl accumulation in the cells studied does depend on the malignant nature of the cells. It is therefore reasonable to conclude that Fl accumulation observed in clinical investigations of human gastric cancer is governed by the pH gradient occurring between the cells and the extracellular fluid; this gradient is much greater in a tumor than in the normal tissues.

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