FLUORESCENCE DYE AS MONITOR OF INTERNAL pH IN *ESCHERICHIA COLI* CELLS

Emanuel SHECHTER, Lucienne LETELLIER and Elisabeth R. SIMONS+

Laboratoire des Biomembranes, Département de Biochimie et de Biophysique, Université de Paris-Sud, 91405 Orsay, France and *+Department of Biochemistry, Boston University School of Medicine, Boston, MA, USA*

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1. Introduction

The most widely used techniques for the determination of intracellular pH are based upon the distribution of an isotopically labeled or fluorescent weak acid or weak base between the intracellular space and the external medium [1,2]. While this technique is direct, it is not always the method of choice [3], particularly for rapid or transient pH changes where equilibration time can be limiting and/or the need to isolate the cells from their medium before each measurement constitutes a time problem. Measurement of intracellular pH via ^{31}P nuclear magnetic resonance of the pH-dependent chemical shift [4–6] is not always applicable to rapidly changing systems.

Techniques which utilize the pH dependence of certain fluorescent or absorbent probes have been developed [3,7–12]. These spectral changes are rapid, can be monitored continuously, and therefore are applicable to dynamic studies. Some of these studies utilize probes such as fluorescein which have been covalently bound to an inert carrier [11], while others [7,9,10,12] rely upon the technique in [7]; the generation of a pH-sensitive fluorescein derivative in situ by intracellular esterolysis of a non-fluorescent precursor. The fluorescent product, e.g., 6-carboxyfluorescein, diffuses much more slowly across the cellular membrane than 6-carboxyfluorescein diacetate, its parent compound [7,8].

The latter technique, developed for cells in culture [7,11], has been used to investigate the pH_{in} of human platelets, and its increase upon stimulation with thrombin [9,10]. While this approach is satisfactory for cells with acidic pH_{in}, the low p*K* of 6-carboxyfluorescein, ~6.5 [7] and consequent insensitivity to pH above 7.4, make the probe less amenable for the determination of alkaline pH_i values.

This is a modification of the original technique allowing the determination of such alkaline intracellular pH_i. It uses the strong pH-dependence of the rate of intracellular hydrolysis of 6-carboxyfluorescein-diacetate by non-specific esterases. We have applied this technique to the determination of pH_i of *E. coli* cells, which is known to be alkaline [4,13,14].

2. Materials and methods

Escherichia coli cells (ML-30, B and K-12) were grown as in [15]. They were treated with 1 mM EDTA to permeabilize the membranes to ionophores (nigericin and valinomycin) [15].

Periplasmic enzymes were released from the cells, *E. coli* ML 308 grown on minimal medium TEA [16] by osmotic shock, as in [17]. The concentrations of enzymes from the periplasmic space (alkaline phosphatase) and from the cytoplasm (β-galactosidase) were evaluated as in [17].

Sonication of the bacteria was performed on ice using a Branson sonicator (25 W for 4 × 30 s). The supernatants were obtained by centrifugation of the sonicates.

Bacteria were suspended in a 1 cm square cuvette at 5×10^8 cells/ml in 5 mM Mes–5 mM Tris–HCl buffer containing 50 mM choline and 100 mM KCl at the desired pH. Wavelength settings were: $\lambda_{exc} = 470$ nm; $\lambda_{em} = 520$ nm on a Perkin-Elmer MPF 44A spectrofluorimeter.

6-Carboxyfluorescein diacetate and 6-carboxyfluorescein were purchased from Molecular Probes, Inc. Nigericin was the kind gift of Dr Nils Bang of Eli Lilly Inc. All other chemicals were of reagent grade and were purchased from commercial sources.

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3. Results

3.1. *pH-dependence of the rate of enzymatic hydrolysis of the dye as measured by the rate of fluorescence change in the presence of sonicate*

Escherichia coli (10^{11} cells/ml) were sonicated and centrifuged as above. An aliquot of the supernatant (5–50 μl) was added to 3 ml 6-carboxyfluorescein diacetate (final conc. 3 μM in buffer at the appropriate pH) and the fluorescence changes recorded continuously. The fluorescence increased linearly with time and, in the pH-range tested (5.5–8.3), the rate of change increased as the pH increased. At every pH, these rates are larger than those observed in the absence of the sonicate supernatant. The difference, V^F (sonicate), is therefore attributable to enzymatic hydrolysis by esterases present in the supernatant, and is shown in fig.1 as a function of pH. In the concentration range tested it is proportional to the amount of esterases, i.e., to the volume of sonicate supernatant added (not shown).

The pH-dependence of V^F (sonicate) (fig.1) is a composite of the pH-dependence of the fluorescence intensity of the hydrolysed dye at the pH in question and of the pH-dependence of the rate of hydrolysis.

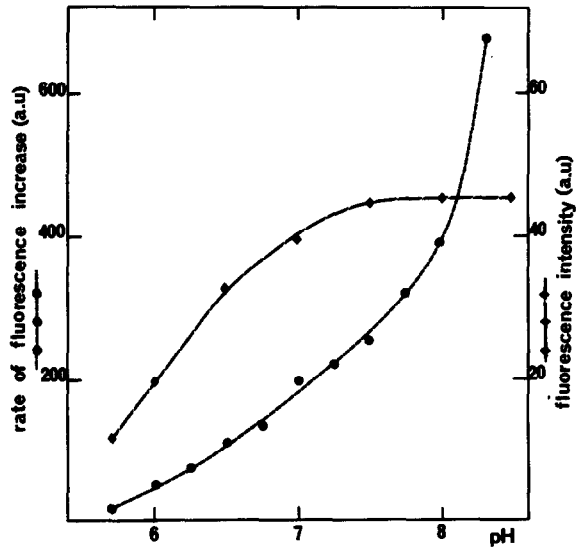

Fig.1. pH-dependence of the rate of fluorescence increase as the result of the hydrolysis of 6-carboxyfluorescein diacetate by the esterases present in the supernatant of the sonicate of *E. coli* cells K-12 (•——•). The data are corrected for the non-enzymatic spontaneous hydrolysis of the dye. pH-dependence of the fluorescence intensity of 6-carboxyfluorescein (♦——♦).

The pH-dependence of the fluorescence intensity in [7,9,10] is confirmed in fig.1. It ceases to be significant at pH > 7.5. Utilization of that intensity thus limits the determination of internal pH to acidic or near neutral values. However, as also shown in fig.1, the pH-dependence of V^F (sonicate), and thus the pH-dependence of the rate of enzymatic hydrolysis, not only extends to alkaline pH-values but increases at pH > 7.5. This makes it possible to extend the determination of internal pH into these regions.

3.2. *Rate of fluorescence change of the dye in the presence of the cells*

A suspension of *E. coli* (5×10^8 cells/ml) was incubated at the appropriate pH in a cuvette, 10 μl 6-carboxyfluorescein diacetate (3 μM final conc.) were added, and the fluorescence change was recorded continuously.

Fig.2 shows the time course of the ensuant fluorescence increase for various external pH-values (6,7,7.5, 8.3); that recorded in the absence of cells was very

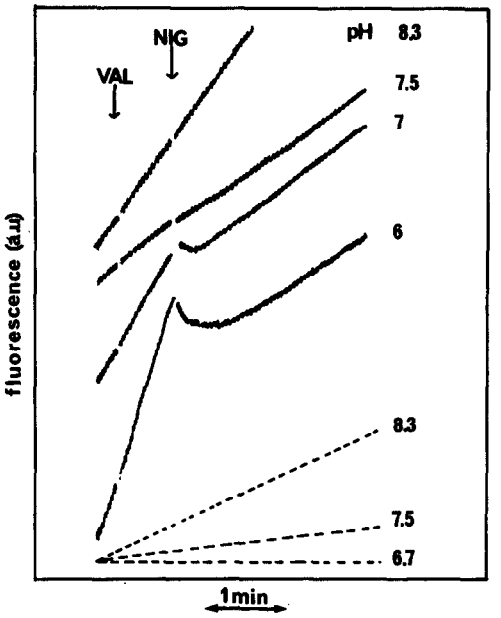

Fig.2. Time course of the fluorescence change as a result of the enzymatic hydrolysis of 6-carboxyfluorescein diacetate in the presence of *E. coli* cells at various external pH-values. Valinomycin (VAL, final conc. 5 μM) and nigericin (NIG, final conc. 5 μM) were added where indicated. The time course of fluorescence change in the absence of cells (spontaneous non-enzymatic hydrolysis) is shown in the lower part of the figure (– – –).

much smaller, and is shown on the lower part of the figure. The larger values observed in the presence of cells indicated that the ester had entered the cell and was being hydrolysed by the internal esterases.

The difference between the rate of fluorescence increase in the presence and in the absence of cells, V^F (cell), was calculated from the above data. Addition of valinomycin, which collapses the transmembrane electrical potential [18], had no effect on V^F (cell). Nigericin, which under our experimental conditions ($K^+_{in}/K^+_{out} \sim 1$) collapses the pH gradient [7,18], led to a decreased V^F (cell) at low pH (fig.2). This effect diminishes as the external pH was increased, and disappeared at pH >7.5. The difference between the rate of probe hydrolysis in the presence of cells and nigericin and that in the absence of cells will be defined as V^F (cell + Nig.) We observed similar results for the *E. coli* strains K-12, ML-30 and B used.

3.3. *Location of the esterases*

E. coli cells possess a periplasmic space located between the inner and outer membranes. To correlate the observed rate of fluorescence increase with cytoplasmic pH it was important to determine whether or not esterases are present in this periplasmic space.

Periplasmic enzymes were released by osmotic shock as in [17]. Alkaline phosphatase, a periplasmic enzyme, was used as a marker for this release. The absence of a concomitant release of cytoplasmic enzymes was verified by monitoring the activity of β-galactosidase, a cytoplasmic enzyme.

Table 1 shows the comparison for *E. coli* ML 308, between the rates of fluorescence increase of the dye in the supernatant of EDTA-treated cells (no enzymes released), of osmotically shocked cells (release of periplasmic enzyme) and of sonicated cells (release of periplasmic and cytoplasmic enzymes). The data clearly show that the esterases responsible for the probe hydrolysis are mainly located in the cytoplasmic space. We assume the same to be true for the other *E. coli* strains used here.

Table 1
Phosphatase alkaline activity, β-galactosidase activity and esterase activity in the supernatants of EDTA-treated cells, osmotically shocked cells and sonicated cells

	Supernatant of		
	EDTA-treated cells	Osmotically shocked cells	Sonicated cells
Phosphatase alkaline activity	15	110	100
β-Galactosidase activity	<1	<1	100
Rate of fluorescence increase of the dye	6	12	100

Phosphatase alkaline activity and β-galactosidase activity were determined as in [17]. The rate of fluorescence increase reflects the concentration of esterases present in the various supernatants. All the data are relative to those obtained in the supernatant of sonicated cells (arbitrarily fixed at 100)

3.4. *Calculation of the internal pH at various external pH-values*

At a given external pH, V^F (cell) will not only be a function of the rate of hydrolysis of the internalized probe by the esterases (i.e., of the internal pH), but also of the internal concentration of the non-hydrolysed ester. The latter will depend on its rate of entry. The non-hydrolysed ester possesses a carboxyl group, and therefore enters the cell more readily at low pH, in its protonated non-charged form [7]. Thus the rate of entry must depend on the external pH, and will be higher, the lower this pH.

It is possible to eliminate the dependence of the rate of entry upon V^F (cell) by considering, at a given external pH, the ratio of V^F (cell) for 2 different internal pH-values, and proceeding as follows: At a given external pH, one can calculate the ratio V^F (cell)/V^F (cell + Nig.). In the absence of nigericin the pH is the unknown to be determined, while in the presence of nigericin the internal pH is equal to the known external one. Thus, the ratio V^F (cell)/V^F (cell + Nig.) depends only upon the relative rate of hydrolysis of the internalized probe by the esterases at the 2 different internal pH-values. It should be equal to the ratio of the rate of hydrolysis of the dye by the esterases in the supernatant of the sonicate at these pH-values:

$$\frac{V^F\,(\text{cell})}{V^F\,(\text{cell + Nig.})} = \frac{V^F\,(\text{sonicate, pH}_1 = \text{internal pH})}{V^F\,(\text{sonicate, pH}_2 = \text{external pH})}$$

The external pH is known. The ratio, V^F (cell)/V^F (cell + Nig.) can be determined experimentally. The internal pH can then easily be calculated from the data in fig.1. An example of such calculation is given in table 2.

Table 2
Internal pH as a function of the external pH for 3 different *E. coli* strains

External pH	Internal pH		
	E. coli K-12	*E. coli* B	*E. coli* ML-30
5.5	7.6		
6.0	7.8	7.5	7.5
6.5	7.8	7.5	7.4
7.0	7.9	7.5	7.5
7.5	7.9	7.5	7.5
8.0		8.0	8.0
8.3	8.3	8.3	8.3

Example of calculation of the internal pH: external pH 6 (*E. coli* K-12); from the data in fig.2, V^F (cell)/V^F (cell + Nig.) = 6.5; from the data in fig.1 at pH 6, V^F (sonicate) = 13; the pH for which V^F (sonicate) equals 6.5 × 13 (84.5) is 7.8, which corresponds to the internal pH

The values of the internal pH, calculated as described above, of *E. coli* strains K-12, ML-30 and B, as a function of the external pH are displayed in table 2. In all cases the internal pH remains nearly constant at pH ~7.5 for an external pH 5.5–7.5. For external pH >7.5, ΔpH becomes negligible and the internal pH increases in parallel with the external one.

Our data are in agreement with those reported by other techniques, i.e., accumulation of weak acids and ^{31}P NMR. This method should prove useful for continuous monitoring of transient and/or rapid changes of intracellular pH-values.

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