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Electrodiffusion of ethidium cation into *Micrococcus luteus* cells

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Abstract

Ethidium bromide fluorescence increased in the presence of *Micrococcus luteus* cells; this was shown to be due to the interaction of the ethidium cation (Eth) with intracellular nucleic acids. Eth permeation across the cytoplasmic membrane was the rate-limiting step and obeyed first-order kinetics. Both the rate of influx and the amount of Eth in cells depended on respiration and on ATPase activity under aerobic and anaerobic conditions, respectively. The initial rate of uptake positively correlated with the membrane potential and was a linear function of Eth concentration in the range from 2 μM to 1 mM. The data indicate electrodiffusion of Eth into *M. luteus*.

Key words: Ethidium bromide; Fluorescent probe; Membrane-potential-dependent transport; Electrodiffusion; (*M. luteus* VKM B-1314)

1. Introduction

The cationic dye ethidium bromide (1,7-diamino-10-ethyl-9-phenylphenanthridinium bromide) can be used as a fluorescent probe since the fluorescent parameters of the Eth cation are dependent on the polarity of the microenvironment [1]. The ability of the probe to intercalate between base pairs in double-stranded regions of nucleic acids has been applied in molecular biology research [1,2]. Eth fluorescence is also known to increase after interaction with mitochondrial and erythrocyte membranes and micellas of anionic detergent [3].

The problem of Eth permeation via cytoplasmic membranes, specifically via microbial ones, is of special interest. The fluorescent responses of the probe in suspensions of *Saccharomyces cerevisiae* cells are attributed to its permeation and interaction with the intracellular nucleic acids [4,5]. An energy dependent uptake linked to the potassium transport system is postulated as a means of the Eth permeation into yeast cells [4,6].

Microspectrofluorimetric investigations of some Gram-positive (*Bacillus subtilis, Staphylococcus aureus*) and Gram-negative (*Serratia marcescens, Enterobacter cloacae*) bacteria have revealed that the former but not the latter were stainable by Eth [7]. Permeabilization of the Gram-negative bacteria by heating made them stainable by Eth. The authors suggested application of the probe for differentiating of Gram-positive and Gram-negative bacteria. This generalization is not substantiated as yet, as Gram-positive mycobacteria were shown to be stained by Eth only after autoclaving [8], i.e., after possible permeabilization.

In resting wild-type *Escherichia coli* K-12 cells, nucleic acids appeared to be accessible to Eth only if the permeability barrier of their envelopes was somehow impaired. The apparent impermeability of the *E. coli* envelope to Eth might be considered to be due to both barrier properties of the outer membrane [9,10] and the work of the unspecific organic cation efflux system of the cytoplasmic membrane [10,11].

In the course of fluorimetric investigations of Eth interaction with bacteria from different taxa (both Gram-positive and Gram-negative), we found that in most cases there is poor, if any, fluorescent response of the probe unless permeability barriers of the cells were

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CTAB, cetyltrimethylammonium bromide; DCCD, *N,N'*-dicyclohexylcarbodiimide; Eth, ethidium bromide; Rh, rhodamine 123.

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impaired by detergent treatment (Puchkov E.O. and Melkozernov A.N., unpublished data). An unusual reaction is observed in the case of *Micrococcus luteus* and some representatives of bacilli. Eth interaction with these bacteria results in a significant increase of its fluorescence.

In the present work this fluorescence effect was further investigated on *M. luteus*. It is shown that Eth permeates into this bacterium by electrodiffusion and that much of the probe is bound intracellularly to nucleic acids.

2. Materials and methods

Reagents. Ethidium bromide (Eth), carbonylcyanide *m*-chlorophenylhydrazone (CCCP), *N,N'*-dicyclohexylcarbodiimide (DCCD), and sodium dodecylsulphate (SDS) were purchased from Serva Finebiochemica, Heidelberg, Germany. Rhodamine 123 (Rh) and cetyltrimethylammonium bromide (CTAB) were from Sigma, St. Louis, MO. Commercially available reagents were used without further purification.

Bacterial strain and growth conditions. The type strain of *Micrococcus luteus* (VKM B-1314) was grown in the modified synthetic medium [12] containing per liter the following: NH_4Cl, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; Na_2HPO_4, 0.2 g; NaH_2PO_4, 0.09 g; KCl, 0.04 g; $CaCl_2 \cdot 2H_2O$, 0.015 g; thiamine-HCl, 0.034 g; glycine, 1 g; sodium glutamate, 5 g; microelements - $MnSO_4 \cdot 5H_2O$, 10 μg; $FeSO_4 \cdot 7H_2O$, 0.001 g; MoO_3, 10 μg; $ZnSO_4 \cdot 7H_2O$, 70 μg; H_3BO_3, 10 μg; $CuSO_4 \cdot 5H_2O$, 5 μg (pH 7.0).

Bacteria were cultivated for 24 h at 37°C with agitation (200 rpm) up to an optical density of 0.2 at 600 nm. Cells were harvested by centrifugation at 4000 $\times g$ for 30 min. Cells were resuspended in the growth medium void of carbon and energy sources (glycine and sodium glutamate) or in 20 mM Tris-HCl buffer (pH 7.0).

Fluorescence measurements. Fluorescence was measured on a Hitachi-850 spectrofluorophotometer (Hitachi, Japan) with a thermostatted cell holder in a quartz cuvette with a 1 cm light path. All measurements were carried out in 3 ml of 20 mM Tris-HCl buffer (pH 7.0), at 37°C. Eth fluorescence was excited at 500 nm and recorded at 600 nm. Eth fluorescence excitation spectra were recorded at 600 nm with 10 μM of Eth. Rh fluorescence was excited at 480 nm and recorded at 520 nm. The slit's width was 5 nm throughout.

Measurements of initial rates of Eth influx. Initial rates of Eth influx were computed from data of Eth uptake measurements. Bacterial suspension (5 mg of dry weight per ml) was added to the solutions of different Eth concentrations in 20 mM Tris-HCl buffer (pH 7.0). After incubation for 2 min at room temperature (20–22°C) the suspension was centrifuged at 10000 $\times g$ for 30 s. The amount of entrapped probe was estimated by evaluating the decrease of Eth concentration in the supernatants. Eth concentration was measured by fluorimetry after adequate dilutions at concentrations of Eth exceeding 25 μM. An appropriate calibration curve was used.

Evaluation of membrane potential changes. For evaluation of the electric membrane potential changes in *M. luteus* a semiquantitative approach based on Rh fluorescence quenching [13] was used. According to this approach, the degree of quenching reversible by uncoupling corresponds to the full scale of membrane potential changes from the initial value down to zero. As an uncoupler 15 μM CCCP was used. Changes of membrane potential were evaluated in relative units.

Oxygen and bacterial respiration measurements. Oxygen content and bacterial respiration were measured as described in [14] with a Clark-type electrode and polarograph PA-2 (Laboratorni Pristroje, Prague) in a 0.7 ml thermostatted cell at 37°C.

In experiments illustrated by Figs. 1 and 3, anaerobic conditions were produced by nitrogen bubbling or due to cells' respiration in closed volume. The time of anoxia was estimated by oxygen measurements in parallel samples. DCCD was added under a stream of nitrogen. A portion of O_2 was added by air sample stirring.

3. Results and discussion

Eth permeation into the cells and interaction with nucleic acids

The kinetics of Eth fluorescence intensity upon the addition of *M. luteus* cells is depicted in Fig. 1A (trace 1). (The same fluorescent response was observed both in growth medium and in 20 mM Tris-HCl buffer (pH 7.0), and, therefore, further experiments were conducted with the buffer).

The fluorescence excitation spectrum of Eth in the bacterial suspension, as compared to the spectrum of Eth in the buffer, was characterised by the increase of the fluorescence intensity at 285 nm and the formation of a well expressed shoulder at 260 nm (Fig. 2, spectrum 2). The Eth spectrum in a mixture with SDS micellas (the latter can be considered as a simplified biomembrane model [3]) had a narrower symmetric band centered at 290 nm (Fig. 2, spectrum 3). According to Le Pecq and Paoletti [1], the appearance of the 260 nm band in the Eth fluorescence excitation spectrum is interpreted as a consequence of energy transfer from bases of nucleic acids to intercalating probe. Hence, the observed increase in the fluorescence of Eth in *M. luteus* cell suspension should be mainly, if

not completely, the result of Eth influx into the cells and its interaction with nucleic acids.

It was shown earlier with Eth that CTAB, a cationic detergent, effectively impairs the permeability barriers of both outer and cytoplasmic membranes in *E. coli* cells [15] and in many other bacteria (Govorunov, I.G., personal communication; Puchkov, E.O. and Melkozernov, A.N., unpublished data). Addition of CTAB to *M. luteus* cells after the Eth fluorescence climax led to the partial decrease of fluorescence (Fig. 1A, trace 1). Treatment of the cells by the detergent prior to Eth addition or right after it resulted in more rapid fluorescence increase but up to a lower level (Fig. 1A, trace 2). At the concentrations used, CTAB did not change Eth fluorescence in solution.

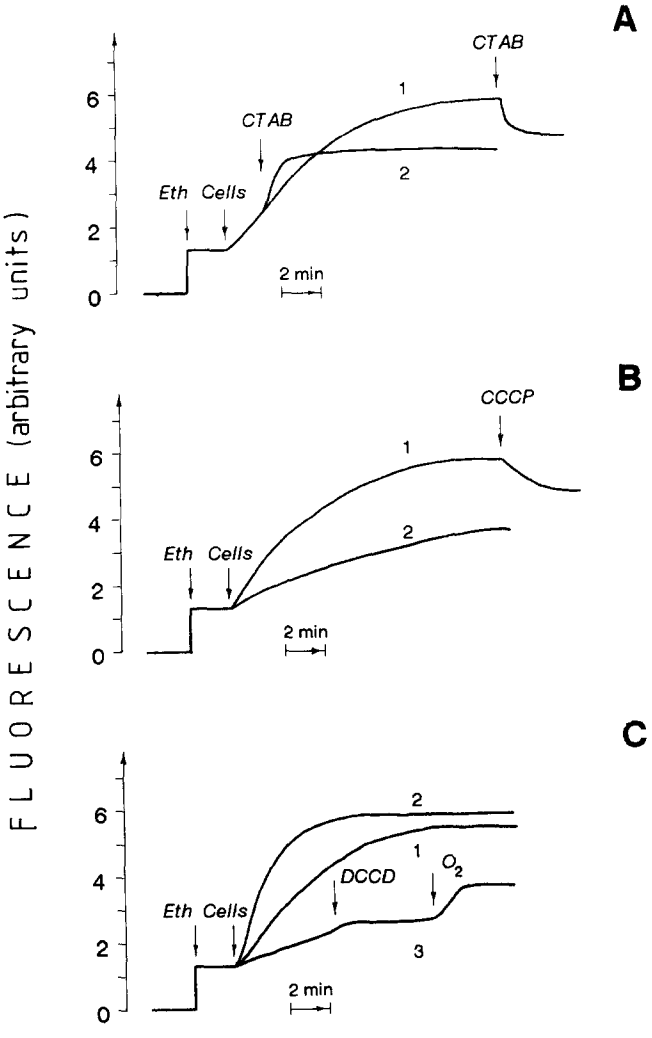

Fig. 1. Time course of Eth fluorescence intensity upon interaction with *M. luteus* cells. (A). Effect of CTAB. (1) Native cells with 100 μM CTAB added after fluorescence response climax; (2) native cells with 100 μM CTAB added at the moment indicated by an arrow. (B) Effect of CCCP. (1) Native cells with 15 μM CCCP added after fluorescence response climax; (2) cells after 10 min incubation with 15 μM CCCP. (C) Effect of DCCD. (1) Native cells; (2) cells after 2 min of aerobic incubation with 100 μM DCCD; (3) native cells under anaerobic conditions with 100 μM DCCD and O_2 added at the moments shown by arrows. Cell concentration was 0.4 mg of dry weight per ml, Eth concentration was 10 μM. Experimental procedures were as described in Materials and methods. Typical results of five experiments are presented.

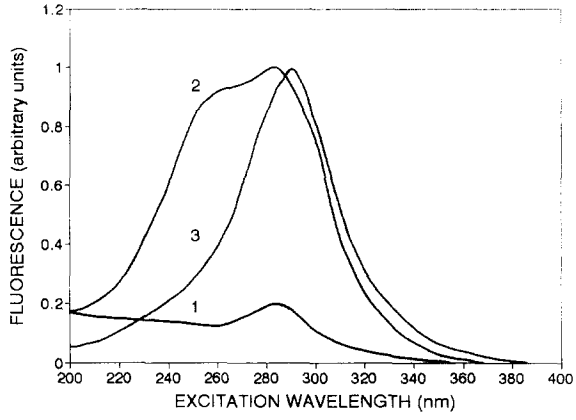

Fig. 2. Fluorescence excitation spectra of Eth: (1) in buffer; (2) in suspension of *M. luteus* cells (0.5 mg of dry weight per ml) after incubation for 20 min; (3) in suspension of SDS (5 mM) micellas. Spectra (2) and (3) were normalized. Spectra were recorded as described in Materials and methods.

Eth binding to nucleic acids is noncovalent. The equilibrating concentration of the probe determines both the amount of bound Eth and the resulting fluorescence increase [1,10]. Taking this into account, the effects of CTAB could be explained assuming that in intact cells intracellular concentration of Eth might exceed that in the medium. In other words, Eth might be concentrated in intact cells. Making cytoplasmic membranes freely permeable for Eth, CTAB induced partial release of accumulated Eth down the concentration gradient. If the detergent had been added before the probe, it accelerated Eth influx.

Membrane potential and respiration dependence of Eth accumulation

Treatment of the cells by an uncoupler CCCP at concentration discharging membrane potential (Fig. 3, trace 1) slightly increased respiration rate (not shown) and induced partial release of the accumulated Eth (Fig. 1B, trace 1). In CCCP pretreated cells both the rate of Eth influx and the amount of permeated probe appeared to be markedly lower (Fig. 1B, trace 2). Also Eth influx is strongly inhibited by anoxia (Fig. 1C, trace 3), when respiration ceases completely (not shown), but comparatively small membrane potential drop is observed (Fig. 3, trace 2). Thus, it was shown that both membrane potential and respiration were essential to Eth accumulation. Eth itself at concentrations of 10–50 μM slightly stimulated respiration and increased the membrane potential (not shown).

DCCD, an inhibitor of the membrane proton-translocating ATPase, caused qualitatively different effects

on Eth transport depending on the oxygen presence in the medium. In aerobic conditions the effect was stimulating (Fig. 1C, trace 2) while in anoxia it was inhibiting (Fig. 1C, trace 3). Evaluation of the influence of DCCD on the membrane potential revealed that it was being increased by DCCD treatment in an air saturated medium and was being decreased in an oxygen-free one (Fig. 3, trace 2).

Since DCCD is known to inhibit proton translocation via proton-translocating ATPase [16], the increase of the membrane potential after DCCD treatement in aerobic conditions might be a consequence of cessation of the partial return to the cytoplasm via ATPase of protons continuously expelled by the respiratory chain [17]. In anoxia the ATPase works as a hydrolase expelling protons and thus sustaining the membrane potential. So, in this case, DCCD reduces the membrane potential. In this context, the effects of DCCD on Eth accumulation (Fig. 1C) support the conclusion on the membrane potential dependence of the probe transport.

Electrodiffusion as a mechanism of Eth accumulation

The time course of Eth interaction with the cells (Fig. 1A, trace 1) fits first-order kinetics. Since interaction of the probe with nucleic acids in CTAB pretreated cells was much faster than in intact cells (Fig. 1A, trace 2), the permeation across the cytoplasmic membrane was a rate limiting step fitting first-order kinetics. This indicates that the translocation process did not involve a carrier.

The initial rate of Eth influx was a linear function of the probe concentration in the range from 2 μM to 1 mM (Fig. 4). These data show that Eth enters the cells by simple diffusion across the cytoplasmic membranes.

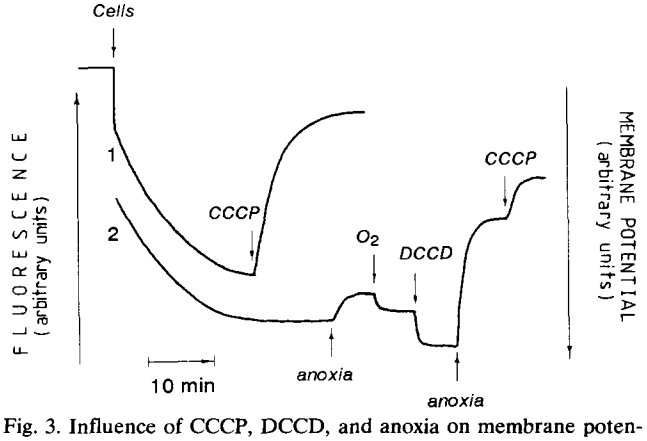

Fig. 3. Influence of CCCP, DCCD, and anoxia on membrane potential in *M. luteus* cells. Curves 1 and 2 represent two separate experiments. The moments of additions of 15 μM CCCP, 100 μM DCCD and the beginning of anoxia are shown by arrows. Concentration of cells was 0.4 mg of dry weight per ml, concentration of Rh was 1 μM. Experimental procedures were as described in Materials and methods. Typical curves of five experiments are presented.

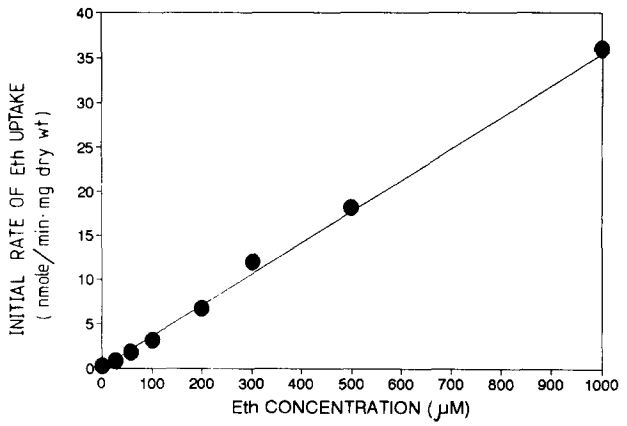

Fig. 4. Dependence of initial rate of Eth influx into *M. luteus* cells on Eth concentration. Initial rates of Eth influx were estimated as described in Materials and methods. Results are expressed as the mean of three separate experiments. S.D. did not exceed 5%.

The dependence of Eth uptake on membrane potential discussed above provides evidence that the accumulation of this cationic probe inside cells could be driven by the membrane potential, inside negative. Hence, according to Kotyk and Janacek [18], it would be better to classify the Eth transport as electrodiffusion.

The rate of electrodiffusion must be a linear function of electric potential gradient according to the following equation [18]:

$$\Phi = -(RTU\partial c/\partial x) - (zFcU\partial\psi/\partial x)$$

where Φ is the flux of an ion; R, gas constant; T, temperature, K; U, diffusion mobility; z, number of elementary charges carried by the ion; F, Faraday constant; c, ion concentration; $\partial c/\partial x$, unidirectional concentration gradient; $\partial\psi/\partial x$, membrane potential gradient across the membrane. Linear correlation between the initial rate of Eth uptake and membrane potential was found to take place in *M. luteus* (Fig. 5).

Since the electrical capacity of the cytoplasmic membrane is low, Eth accumulation in *M. luteus* might take place provided counterflow of protons could be effectively sustained by the respiratory chain or ATPase (Figs. 1 and 3). Eth permeation via other biomembranes must require at least charge compensating fluxes of other ions, i.e., unidirectional or counterdirectional fluxes of anions and cations, respectively. Absence of such fluxes may be one of the reasons of an apparent poor stainability of the cells by Eth. Appearance of Eth permeation across natively impermeable membranes after some treatments may be the consequence of the impairment of the membrane barrier for some other ions.

As mentioned in the Introduction, phenomenology of Eth interaction with bacilli had been similar to that

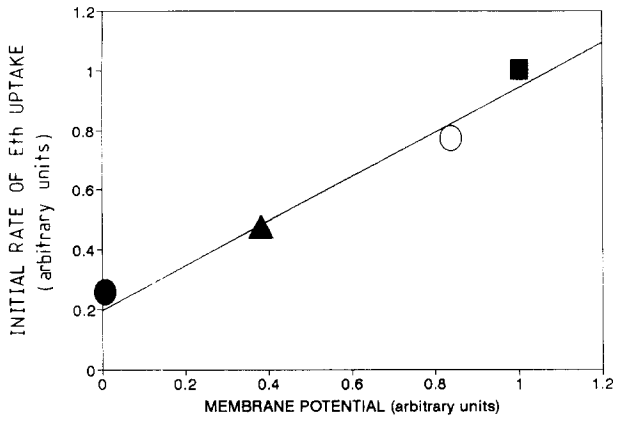

Fig. 5. Correlation between membrane potential and initial rate of Eth influx in *M. luteus* cells. Membrane potential was measured as described in Materials and methods. Initial rates of Eth influx were computed from the linear region of Eth fluorescence time course. Eth concentration was 10 μM. Cell concentration was 0.5 mg of dry weight per ml. ■, no additions; ○, addition of 40 mM KCl; △, addition of 6 μM CCCP; ●, addition of 15 μM CCCP. Each point is the mean of three separate experiments. S.D. was equal to or smaller than the symbols.

for *M. luteus* (data not shown). The accumulation of Eth into cells due to electrodiffusion could have been the cause of hypersensitivity of bacilli [19] and staphylococci [20] to micromolar concentrations of the drug.

Acknowledgments

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