Ethylrhodamine as a fluorescent penetrating cation and a membrane potential-sensitive probe in cyanobacterial cells

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Ethylrhodamine, a homolog of rhodamine 123, was found to generate a diffusion potential across a planar bilayer phospholipid membrane (the compartment with the lower ethylrhodamine concentration positive). Addition of a penetrating synthetic anion increased the sensitivity of the membrane to ethylrhodamine, an effect similar to that previously described for triphenylmethylammonium, tetraphenylphosphonium and other synthetic penetrating cations. In experiments with the filamentous multicellular cyanobacteria *Phormidium uncinatum*, ethylrhodamine was used as a cationic penetrant and vital fluorescent probe for membrane potential ($\Delta\Psi$). It is shown that a treatment increasing protonic potential (illumination) and converting ΔpH to $\Delta\Psi$ (nigericin) enhanced the fluorescence of cyanobacteria, as ethylrhodamine accumulated inside the cells. Uncouplers discharging $\Delta\Psi$ completely abolished the fluorescence increase. Individual variations in the fluorescence of cells composing cyanobacterial trichomes could be observed. Illumination of a small part of a trichome with a narrow beam of the light actuating photosynthesis was shown to induce a fluorescence increase in both illuminated and non-illuminated regions of this trichome, indicating $\Delta\Psi$ transmission from cell to cell.

Membrane potential *Cyanobacteria* *Rhodamine* *Fluorescent probe*

1. INTRODUCTION

In bioenergetics, an important task is to measure the in vivo electric potential difference ($\Delta\Psi$) across energy-transducing membranes. Until recently, the available methods could only be used with suspensions of bacteria so that study on an individual cell was not possible.

Synthetic penetrating ions were introduced in this group [1–3] as $\Delta\Psi$ probes for the in vitro studies on mitochondria, submitochondrial particles and bacterial chromatophores. Later this approach was extended to bacteria, their particles, proteoliposomes, etc. (review [4]). All these studies have led to the conclusions that (i) any vesicle bearing $\Delta\Psi$, inside negative, accumulates cations if the latter can cross the phospholipid bilayer, and (ii) it accumulates anions provided that $\Delta\Psi$ is inside positive. This finding allows a novel approach to the search for vital $\Delta\Psi$-monitoring dyes. In particular, one may hope that penetrating cationic dyes will specifically stain cells of mitochondria since their interior charges negatively. This method was explored in our group by D.B. Zorov (1978, unpublished) and, independently, by Chen et al. [5–7] to stain mitochondria in living animal cells. Derivatives of the fluorescent cation rhodamine were used as $\Delta\Psi$ probes (see [8–11]).

Here, we use the same approach to study systems other than mitochondria. It is found that a gradient of the ethyl ether of rhodamine, a homolog of methylrhodamine (rhodamine 123) applied in [5–7], generates a $\Delta\Psi$ across a planar bilayer phospholipid membrane as if this dye were a penetrating cation. The membrane can be sensitized to a low concentration of ethylrhodamine by adding small amounts of a penetrating anion. Having ascertained that ethylrhodamine is a pene-

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trating cation, we applied it to studying energization of cells of cyanobacteria. The data obtained are consistent with the assumption that ethylrhodamine accumulates inside cyanobacterial cells down the electrical gradient.

2. MATERIALS AND METHODS

The filamentous multicellular cyanobacteria *Phormidium uncinatum* from Lake Baikal were grown as in [12].

One day before the experiment, trichomes of *P. uncinatum* were transferred from liquid medium to a 22 mm^2 cover slip moistened with 0.8 ml medium D (see [12]). Then the slips were stored in a petri dish until microscopic examination. Aqueous stock solution of ethylrhodamine was diluted with medium D to 2.5 μM concentration. The trichomes were incubated with this concentration of ethylrhodamine for 10 min and rinsed with medium D without ethylrhodamine for 1 min. Fluorescence of trichomes was observed under a fluorescent Univar microscope using a 100 W tungsten lamp as light source and a blue Univar filter N49 so that fluorescence was excited with 400–490 nm light and observed at 500–550 nm.

Asolectin planar black membrane was formed in the aperture of a septum separating a Teflon chamber. The diameter of the aperture was 1 mm.

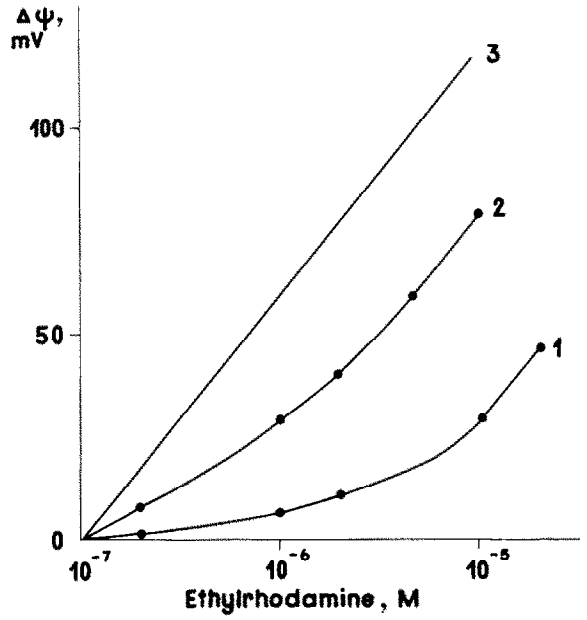

Fig.1. Generation of electric potential difference ($\Delta\Psi$) across a bilayer phospholipid membrane by a transmembrane ethylrhodamine gradient. In one of the compartments separated by the bilayer membrane, the ethylrhodamine concentration was 10^{-7} M. In the other compartment, it was varied (see abscissa). Curve 1, without addition of penetrating anions. Curve 2, 10^{-7} M phenyldicarbaundecaborane was added. Curve 3, theoretical (Nernst) relationship for a monovalent cationic penetrant.

3. RESULTS

Fig.1 shows the results of an experiment with a planar phospholipid membrane. The ethylrhodamine concentration on one side of the membrane was constant and equal to 1×10^{-7} M, that on the other side was varied. It is shown that the transmembrane ethylrhodamine gradient results in the generation of a $\Delta\Psi$. The compartment with the higher ethylrhodamine concentration charges negatively as if a cation moved across the membrane down the concentration gradient.

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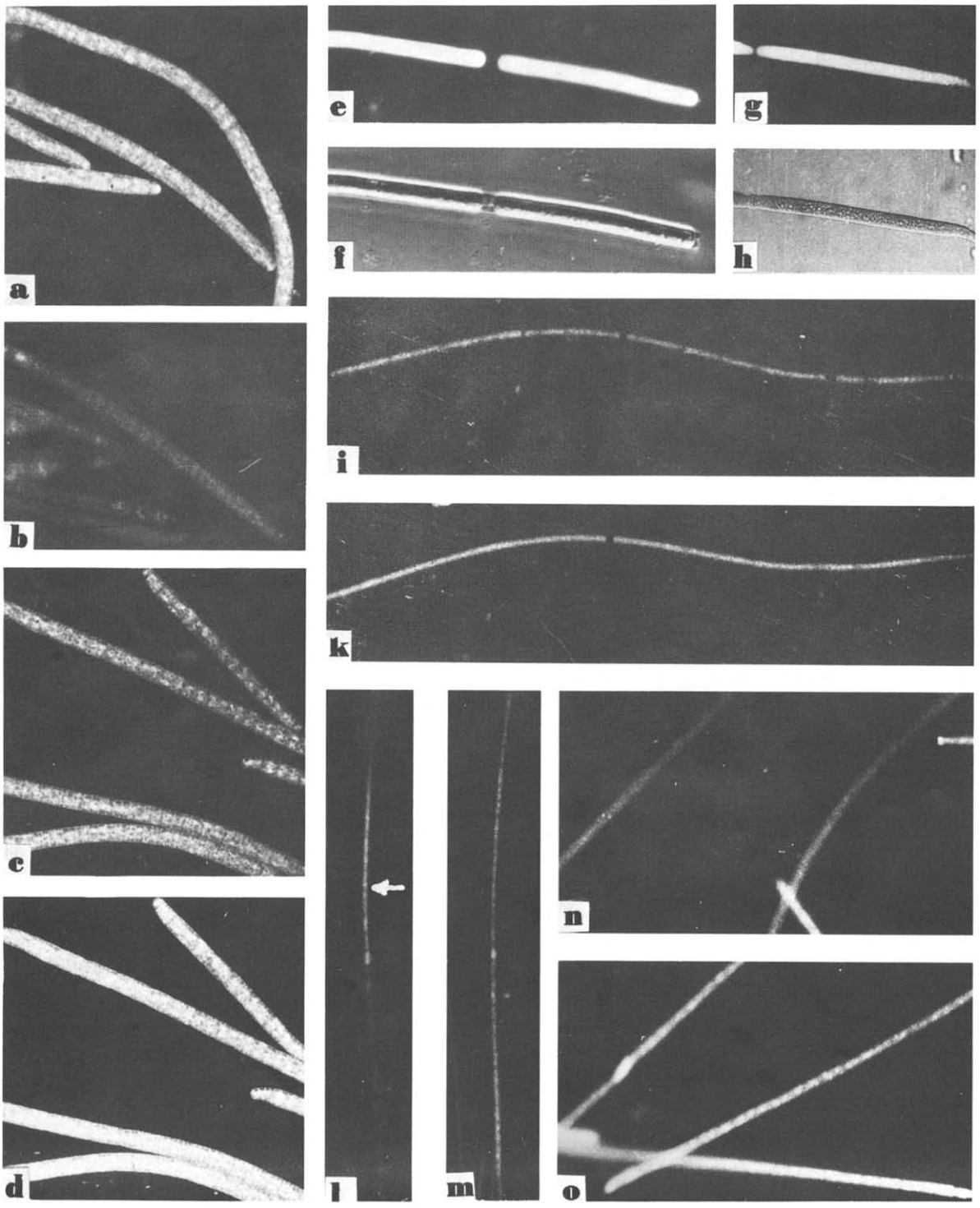
Fig.2. The use of ethylrhodamine as a fluorescent probe for membrane potential in *P.uncinatum* cells. (a,b) *P.uncinatum* trichomes were incubated in medium D containing 2.5 μM ethylrhodamine, without or with 1×10^{-4} M tetrachlorotrifluoromethylbenzimidazole, respectively. Aerobic conditions. Fluorescence microscopy, ×640. (c,d) Trichomes were incubated and photographed under anaerobic conditions in the medium as in a, but supplemented with 0.022 M glucose, glucose oxidase and catalase (0.01 and 0.2 mg/ml, respectively). After incubation for 2 min in the dark, the light was switched on and photographs were made immediately or after 15 s illumination (c and d, respectively). Fluorescence microscopy, ×640. (e–k) Conditions as in d; in (e,g,i) fluorescence microscopy; (f) phase contrast microscopy of the trichomes shown in e; (h) interference contrast microscopy of the trichome shown in g. (k) The same trichomes as in i, but after 8 s light exposure. Fluorescence microscopy. In i and k, arrows indicate places where dark cells became fluorescent after 8 s illumination. Magnifications: (e,f) ×640; (g,h) ×475; (i,k) ×190. (l–n) Conditions as in c,d but



in the dark one of the trichomes was illuminated by a narrow light beam covering a small part (5%%) of its length (arrow). Photographs were made immediately after switching on the total illumination (l) or 8 s later (m). Fluorescence microscopy, ×110. (n,o) Effect of nigericin; (n) without nigericin; (o) with 1×10^{-5} M nigericin; fluorescence microscopy, ×330.

As shown previously [3], addition of a small amount of synthetic penetrating ions sensitizes the planar membrane to penetrating cations. Such an effect was also observed with ethylrhodamine. As the anion, phenyldicarbaundecaborane was used (fig.1).

Thus one may hope that ethylrhodamine as a penetrating cation should be accumulated by energized bacterial cells since their interior charges negatively relative to the outer medium. This suggestion was confirmed in experiments with *P.uncinatum*. It was found that in the light, *P.uncinatum* trichomes incubated with ethylrhodamine emit bright green fluorescence which can be abolished by an uncoupler discharging $\Delta\Psi$ across the cyanobacterial membrane (fig.2a,b). Incubation of trichomes under anaerobic conditions in the dark strongly decreases green fluorescence. Switching on illumination results in an increase in the fluorescence intensity which reached its maximum within 15 s (fig.2c,d).

All fluorescence responses could be greatly accelerated by addition of phenyldicarbaundecaborane (not shown). No green fluorescence was observed in the absence of ethylrhodamine.

As a rule, all cells in a particular trichome showed a similar intensity of fluorescence, which confirms our previous observation indicating that the cyanobacterial trichome usually represents an electrically united system [12–15]. However, sometimes we succeeded in finding a trichome which contained a 'dark' cell(s) displaying a much lower intensity of fluorescence than other cells in the same trichome (fig.2e–g). When scrutinized in transparent light such cell(s) could show (fig.2f,h) morphological differences from or not differ morphologically from neighbouring cells. In the latter case, illumination for a minute usually resulted in the disappearance of the fluorescence differences (fig.2i,k).

Trichomes interrupted with dark cell(s) showed unusual responses to partial illumination. As observed in [12–15], a light spot covering only 4–5%% of the trichome length energizes all the cells composing the trichome; this followed from the $\Delta\Psi$ transmission and motility probes. Experiments on the dark cell-containing trichomes showed that the light spot failed to induce an ethylrhodamine fluorescence increase in that part of the trichome which was separated from the light spot. If this part was longer than that illuminated by the light spot, the trichome was motionless until the total illumination was switched on. These facts can be accounted for by the hypothesis that a dark cell is electrically isolated from its neighbours so that it cannot be involved in the $\Delta\Psi$ transmission along the trichome.

Sometimes, the light spot failed to energize a trichome even if it did not contain the dark cells. An example of this kind is given in fig.2l,m. It is seen that the light spot induced a fluorescence increase in a part of the trichome. The rest became fluorescent only under total illumination.

This observation can be explained by the assumption that electrical contacts between cells, which usually are of low electrical resistance can, under certain conditions, be switched off so that electrical transmission along the trichome is interrupted in some places.

In the last series of experiments, the effect of nigericin was studied. It was found (fig.2n,o) that nigericin added to 1-day-old trichomes increased their ethylrhodamine-linked fluorescence. Without nigericin, some trichomes appeared almost dark. In its presence, practically all the trichomes were fluorescent. Trichomes which were dark without nigericin were motile like those which were fluorescent, indicating that there was a protonic potential across the membrane of dark trichomes. Apparently it was mainly in the form of ΔpH so that the nigericin effect can be accounted for in the $\Delta\text{pH} \rightarrow \Delta\Psi$ transition by the exchange of H^+ for monovalent cations, catalyzed by this antibiotic.

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