MEMBRANE ORIENTATION IN VESICLES FROM *MICROCOCCUS LYSODEIKTICUS* CELLS

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1. Introduction

Knowledge of membrane orientation is of primary importance in elucidation of transport phenomena in closed vesicles. The orientation may be ascertained by means of electrophoretic ion transport, the direction of which depends on the direction of the electric field established on energizing the membrane. Such transport experiments are generally done with the aid of synthetic lipid-soluble anions and cations [1] or of K^+ or Rb^+ in the presence of valinomycin [1,2].

It is now well established that the inner surface of energized bacterial membrane carries a net negative charge [2]. Consequently, vesicles with membranes of the same orientation as the intact cells (rightside-out) should perform energy-dependent accumulation of cations, whereas those with opposite (inside-out) orientation of the membrane-accumulation of anions. Despite the extensive use of membrane vesicles in various experiments [2–7], we have found no investigations of anion and cation transport in one and the same fractions of bacteria, although this could reveal the homogeneity or heterogeneity of the vesicles in a given fraction with respect to membrane orientation.

This we have done in the present study, determining both anion transport (phenyl dicarbaundecaborane anions, PCB^-) and cation transport (K^+ in the presence of valinomycin) in membrane fractions of *Micrococcus lysodeikticus* prepared by osmotic shock and sonication and have found that such fractions are actually mixtures of vesicles with oppositely oriented membranes. The results have been confirmed by an electron microscopic study of osmotically shocked fractions.

2. Materials and methods

The sub-bacterial particles from *M. lysodeikticus* Flemming strain were prepared by osmotic shock or sonication as described by Tikhonova et al. [8].

The incubation medium consisted of 0.25 M sucrose, 0.05 M Tris–HCL (pH 7.5) and 0.005 M $MgSO_4$ to which were added, depending upon the experiment, 0.005 M malate, lactate or succinate, 0.005 M ascorbate + 0.0001 M *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine (TMPD) or 0.001 M NADH as oxidation substrates, 0.005 M NaCN as inhibitor of respiration, and 10^{-6} M carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). PCB^- was added in 10^{-6} M concentration in studies of anion transport and 0.001 M KCl and valinomycin in 10^{-6} M concentration in studies of K^+ transport. The composition of the medium made it possible to follow the transport by determining the changes in concentration of these ions in the medium. Determination of the K^+ concentration was with a K^+-sensitive glass electrode and the PCB^- concentration by a procedure used in similar experiments by Grinius et al. [9], employing a phospholipid bilayer as the selective electrode. The concentration of membrane particles in the medium corresponded to 3–4 mg protein/ml and all the measurements were made in a temperature controlled cell at 26°C.

3. Results and discussion

The results of measurements of the energy-dependent PCB^- uptake in the presence of malate are shown on fig. 1. It can be seen that both the osmotically treated

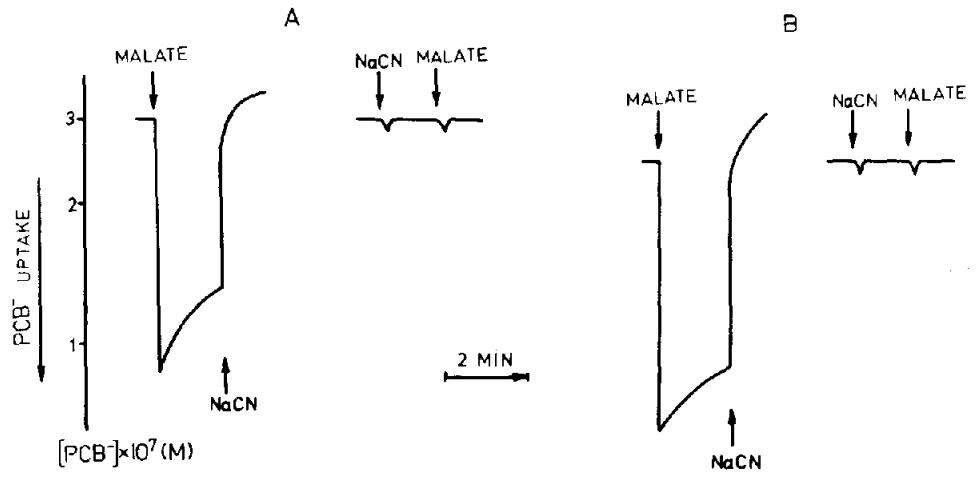

Fig. 1. Energy-dependent PCB^- uptake by membrane fractions prepared A – by osmotic shock; B – by sonication. Incubation mixture: sucrose – 0.25 M; Tris–HCl – 0.05 M (pH 7.5); $MgSO_4$ – 0.005 M; PCB^- – 10^{-6} M; A – 0.5 mg protein/ml; B – 0.6 mg protein/ml. Additions: malate – 0.005 M; NaCN – 0.005 M.

and sonicated membrane fractions accumulate anions and uptake is totally inhibited by cyanide. At the same time cyanide inhibited the respiration of the osmotically treated membrane fractions by 30–40% and of the ultrasonic fractions by 70%. These findings, in complete agreement with the results of Tikhonova et al. [8] and Grinius et al. [9], show that the membrane fractions contain closed inverted vesicles.

Because of the comprehensive nature of the studies by Tikhonova et al. [8] and Grinius et al. [9] we did not go any further into this matter directing our main attention to study of the cation transport.

Contrary to the intact cells, membrane vesicles from *M. lysodeikticus* [10] do not accumulate K^+, the uptake of this cation being observed only in the presence of valinomycin. Data on the energy-dependent K^+ trans-

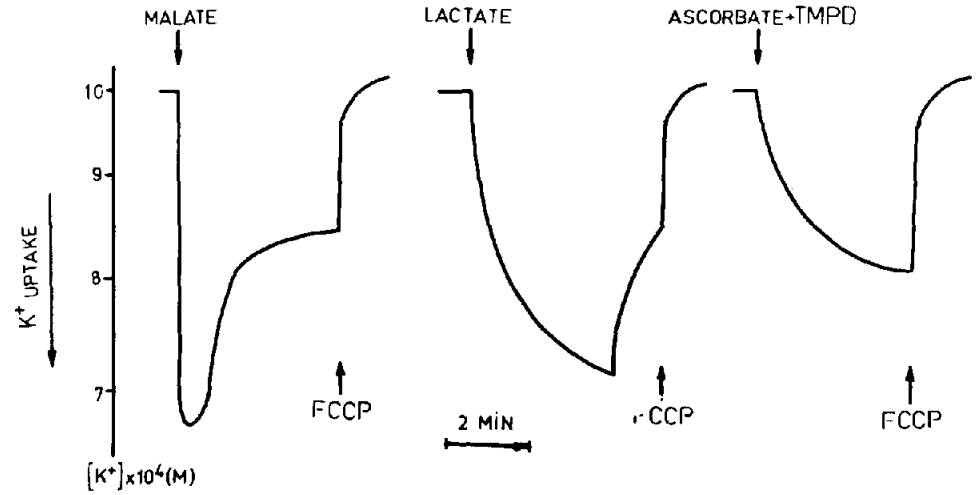

Fig. 2. Energy-dependent K^+ uptake by membrane fractions prepared by osmotic shock. Incubation mixture: sucrose – 0.25 M; Tris–HCl – 0.05 M (pH 7.5); $MgSO_4$ – 0.005 M; KCl – 0.001 M; valinomycin – 10^{-6} M; membranes – 3.8 mg protein/ml. Additions: malate, lactate, ascorbate – 0.005 M; TMPD – 10^{-4} M; FCCP – 10^{-6} M.

Table 1
Respiration of membrane fractions of *Micrococcus lysodeikticus*

Substrate	Respiration rate (natoms oxygen/ min per mg protein)	Per cent of cyanide-sensitive respiration
Malate	340	30–40%
Lactate	170	30–40%
Succinate	10	–
NADH	180	–
Ascorbate + TMPD	75	100%

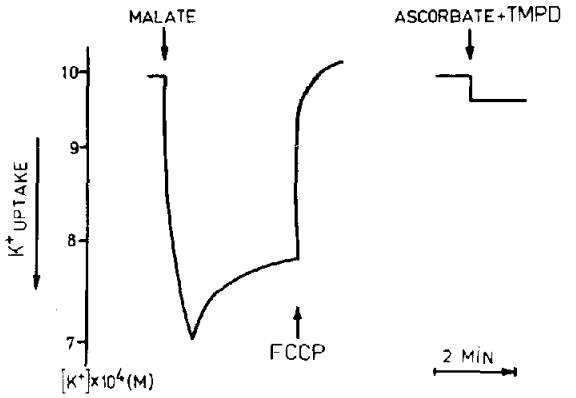

Fig. 4. Uptake of K^+ ions in the presence of cyanide by osmotic shock-prepared membrane fractions. Incubation mixture is the same as in fig. 2, NaCN – 0.005 M.

port in the presence of antibiotic for membrane fractions prepared by osmotic shock are represented on fig. 2. Active K^+ uptake by the vesicles occurred when malate, lactate and ascorbate + TMPD were added. No K^+ transport was observed in the presence of succinate and NADH. The absence of K^+ uptake in the presence of succinate could be ascribed to the slow oxidation of this substrate (table 1), whereas with respect to NADH, since NADH dehydrogenase is known to be located at the inner surface of the intact cell membrane of *M. lysodeikticus* [8], vesicles enclosed by rightside-out membrane apparently could not make use of this non-penetrating substrate. Addition of FCCP, which increases membrane permeability to protons thereby dissipating the electric potential across the membrane, leads to rapid efflux of all the absorbed K^+ (fig. 2). No energy-linked K^+ uptake was observed when FCCP was added to the incubation medium prior to the respiration substrate (fig. 3). It should be noted that K^+ was accumulated by the vesicles in approximately the same amounts on oxidation of malate,

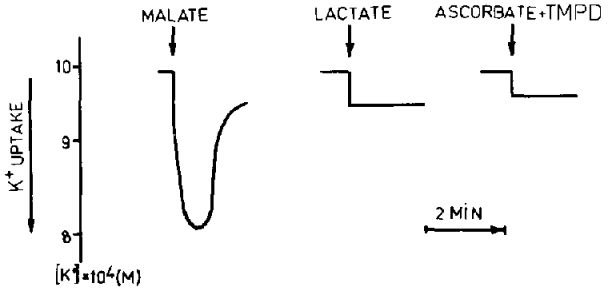

Fig. 3. Uptake of K^+ ions in the presence of a proton-conducting uncoupler by osmotic shock-prepared membrane fractions. Incubation mixture is the same as in fig. 2, FCCP – 10^{-6} M.

lactate and ascorbate + TMPD, although with the last substrate the respiration rate was considerably lower (table 1). The K^+ uptake curves in the presence of malate display a characteristic peak at the onset of the accumulation, not disappearing on addition of uncoupler (fig. 3).

Fig. 4 shows the effect of cyanide on K^+ transport in the vesicles prepared by osmotic shock. Under the experimental conditions the malate-oxidation-coupled K^+ uptake underwent a slight increase, rather than a decrease, in the presence of cyanide (fig. 3). Hence K^+ uptake by the vesicles is associated with cyanide-insensitive malate oxidation, whereas anion uptake is associated with its cyanide-sensitive oxidation. In similar experiments with ascorbate +TMPD as substrate, cyanide totally inhibited both K^+ accumulation and respiration.

As well as the osmotic shock-prepared fractions the sonication-prepared membrane fractions carried out energy coupled K^+ uptake in the presence of valinomycin. However, the amount of absorbed K^+ with respect to the same protein content was much lower in the sonication-prepared, than in osmotic shock-prepared fractions.

The conclusion that the osmotic shock-prepared fractions comprise a mixture of vesicles with oppositely oriented membranes has been substantiated by an electron microscope study of these fractions. Fig. 5 shows an electron micrograph of the two types of vesicles; inside-out vesicles with ATPase subunits on the outer side and rightside-out vesicles wihout the external ATPase subunits.

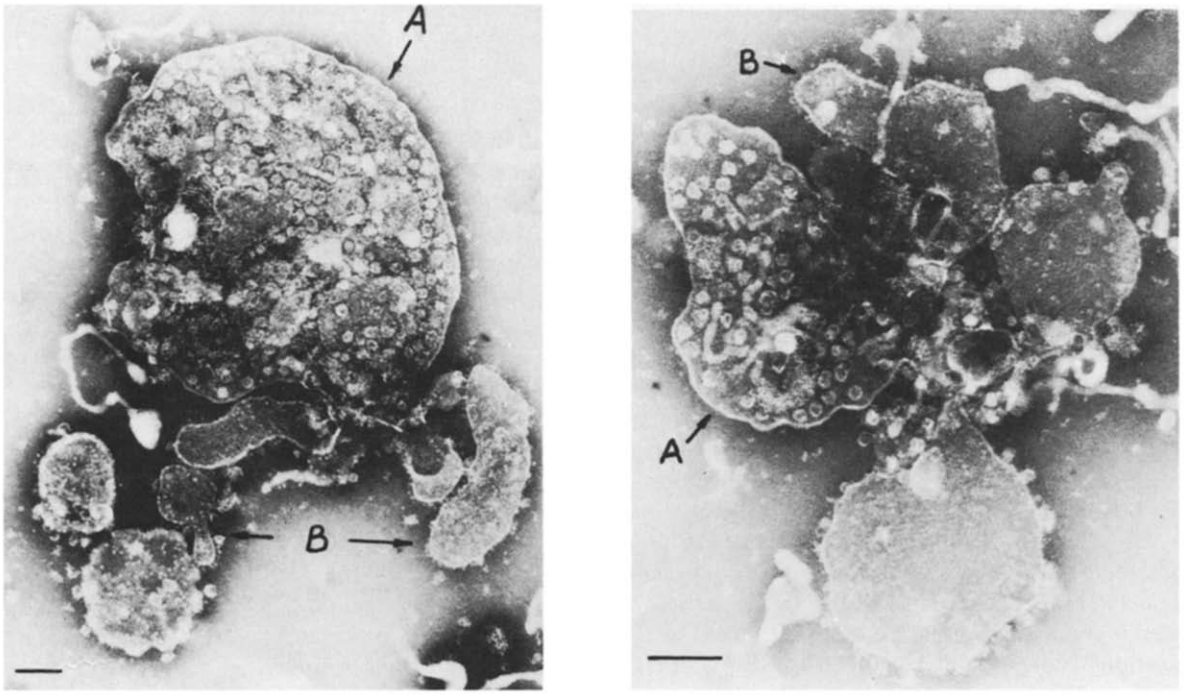

Fig. 5. Electron micrographs of the osmotic shock-prepared membrane fraction of *M. lysodeikticus*. Preparation was negatively stained with 1% phosphotungstate at pH 7.5. The *marker bars* correspond to 2μm. A – Rightside-out vesicle; B Inside-out vesicle.

Acknowledgements

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