DETERMINATION OF THE MEMBRANE POTENTIAL IN BACTERIAL MEMBRANE VESICLES FROM THE ACCUMULATION OF *N*-METHYLDEPTROPINE

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1. Introduction

Active transport of solutes by cytoplasmic membrane vesicles from bacteria can be driven by electron transfer in respiratory chains, anaerobic electron transfer systems or by cyclic electron transfer systems (reviewed [1,2]). Electron transfer results in the generation of an electrochemical proton gradient across the membrane which is composed of an electrical gradient ($\Delta\psi$) and a chemical proton gradient (ΔpH) according to the equation $\Delta\tilde{\mu}_{H^+} = \Delta\psi - 2.3\, RT/F \log \Delta pH$. The generation of a membrane potential and a pH gradient, induced by electron transfer, has been demonstrated in membrane vesicles from several bacteria [3,4].

The transmembrane pH gradient can be determined from the accumulation of radioactively labeled weak acids or bases as measured by means of flow dialysis [5]. For the determination of the membrane potential several methods have been employed [3]. A very reliable method for small cells and membrane vesicles has been introduced [6]. These investigators demonstrated that lipophilic organic cations or anions distribute across the membrane in response to the membrane potential. The membrane potential can be calculated from the concentration gradient reached at steady state level of accumulation with the Nernst equation:

$$\Delta\psi = RT/nF \ln C_{in}/C_{out}$$

in which n = valency of the ion, C_{in} = concentration inside the membrane and C_{out} = the medium concentration of the lipophilic ion.

Membrane potentials (inside negative) have been determined from the accumulation of several lipophilic cations, i.e., dibenzyldimethylammonium (DDA) [7], tetraphenylphosphonium (TPP) [8] and triphenylmethylphosphonium (TPMP) [9]. A considerable disadvantage of all these cations is that they are not commercially available in radioactively labeled form or that complicated procedures are needed in order to synthesize these compounds in radioactive form. Here we describe the use of the lipophilic cation *N*-methyldeptropine (MDT) for $\Delta\psi$ measurements in bacterial membrane vesicles. This compound can be synthesized in radioactive form by a very simple procedure. Two related compounds, *N*-methyltropine (MT) and *N*-methylatropine (NMA) were also investigated to get more information about the properties of an organic cation that determine its permeability across the bacterial membrane and to act as a $\Delta\psi$ probe.

2. Materials

2.1. *Cell growth and preparation of membrane vesicles*

Escherichia coli ML 308–225 was grown under aerobic conditions at 37°C to late logarithmic phase on Davis-Mignoli medium supplemented with 0.1% yeast extract (Difco) and 1% sodium succinate as carbon and energy source. Membrane vesicles were isolated as in [2,10], suspended in 50 mM potassium

phosphate (pH 6.6) and stored in liquid nitrogen. For each experiment vesicle suspensions were used, which were frozen and thawed only once.

2.2. *Transport assays*

The vesicle uptake of radioactively labeled solutes was determined as in [2,9]. Assays were carried out at 20°C. The reaction mixture (100 μl) contained 0.1 mg membrane protein/ml, 50 mM potassium phosphate (pH 6.6), 10 mM magnesium sulphate and electron donor. For energization of the vesicles the non-physiological electron donor potassium ascorbate (10 mM) plus phenazine methosulphate (PMS) (100 μM) was used. Before addition of the labeled solute the reaction mixture was allowed to equilibrate for 2 min. The uptake experiment was started by the addition of the labeled compound. The reaction was stopped by rapid dilution of the reaction mixture with 2 ml 0.1 M LiCl and filtering over cellulose acetate filters (Oxoid nuflow membrane filters N 25/45 UP, pore size 0.45 μm). The internal concentrations of solutes inside the membrane vesicles were calculated using 2.2 μl/mg membrane protein as the intravesicular volume [11].

2.3. *Generation of a valinomycin–potassium diffusion potential*

A suspension of membrane vesicles (1.0 mg/ml) in 50 mM potassium phosphate (pH 6.6) was incubated at room temperature over 5 min with 4 μM valinomycin. The vesicles were washed once and resuspended in the same buffer to a protein concentration of 10, 5 and 1 mg/ml respectively. At zero time these suspensions were diluted 100, 50 and 10 fold, respectively, into a reaction mixture, which contained 50 mM sodium phosphate (pH 6.6), 10 mM magnesium sulphate and the labeled solute to a final volume of 100 μl. A reaction mixture, which contained 50 mM potassium phosphate instead of sodium phosphate was taken as control.

2.4. *Protein determination*

Protein was assayed by the Lowry method [12].

2.5. *Synthesis of radioactively labeled* N-*methyldeptropine and* N-*methyltropine*

Deptropine citrate was purchased from Gist-Brocades Pharmaca, Rijswijk (Z.H.).

^{14}C-Labelled 3α((10,11-dihydro-5H-dibenzo-[a,d]-cyclohepten-5-yl)oxy)-8-methyltropaniumiodide (MDT) (spec. act. 1.21 μCi/μM) was synthesized from deptropine citrate and [^{14}C]methyliodide by the central laboratory of TNO, Delft, by combining equimolar quantities of deptropine citrate and [^{14}C]methyliodide dissolved in acetone. The mixture was incubated at room temperature for 3 days. The acetone was distilled off and the residue dissolved in water. Purity control was performed by thin-layer chromatography (TLC) and scanning. Radiochemical purity was >98%.

N-[^{14}C]methyltropaniumiodide (MT) (spec. act. 1.21 μCi/μM) was synthesized by hydrolysis of [^{14}C]MDT with 10 mM HCl at pH 2. After centrifugation the supernatant contained only MT, a precipitate was formed by the cyclohepten part. The supernatant was neutralized to pH 6.6 with KOH. Purity control was performed by TLC and scanning. Radiochemical purity was >98%. Aqueous solutions of MT and MDT are very stable, but NMA easily decomposes [14].

2.6. *Materials*

Tritium-labeled TPP (spec. act. 50 μC/μM) was kindly donated by Dr H. R. Kaback, Roche Institute of Molecular Biology, Nutley, 07110 NJ. *N*-[^{14}C]-methylatropine (NMA) (spec. act. 6.98 μCi/μM) was a generous gift of Dr A. M. Soeterboek. All other chemicals used were reagent grade and obtained from commercial sources.

3. Results

3.1. *Physicochemical properties*

MDT and NMA are synthetic analogues of atropine and belong to the pharmacologic class of anticholinergics [13,15–17]. MT is the product of hydrolysis of both MDT and NMA. The structures of MT, MDT and NMA are given in fig.1. All three compounds contain the quaternary tropine part; only the chemical structure of the substituted alcohol group of the tropine moiety varies. The partition of MT, MDT and NMA between *n*-octanol and various aqueous phases has been determined as an indication of their lipophilicity (table 1). The partition behaviour of these structurally related compounds differs considerably. Only MDT dissolves well in octanol, indicating that

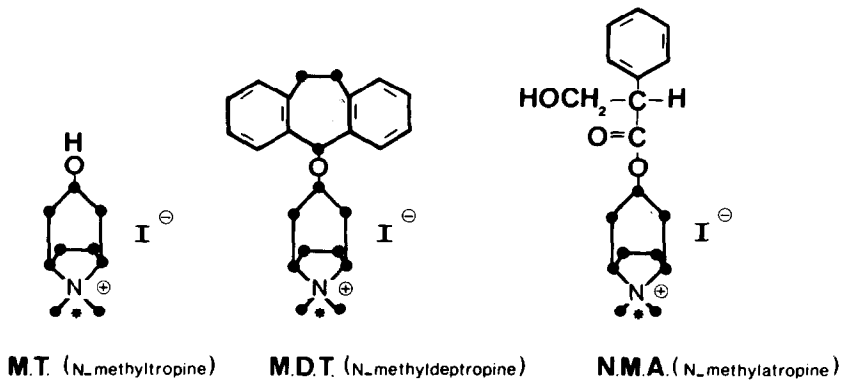

Fig.1. Structure of MT, MDT and NMA. The asterisk shows the position of the label.

Table 1
Partition of MT, MDT and NMA between octanol and various aqueous phases, determined by the rotating flask method

Compound	% dissolved in octanol (pH 7.4)		
	H_2O	Krebs–NaOH	Krebs–bicarbonate
MT	< 0.1	< 1.0	< 1.0
MDT	24.5	88.5	90.8
NMA	0.8	1.6	1.6

MDT is more lipophilic than MT or NMA. This observation shows that the sidegroup of the tropine part determines to a large extent the solubility in octanol. It remains to be established whether ion-pair formation of the particular cations with anions in the media also effects this partition behaviour.

3.2. *Uptake of MDT and TPP by membrane vesicles*

Uptake of MDT and TPP by membrane vesicles of *Escherichia coli* ML 308–225 was observed in the presence of ascorbate-PMS (fig.2). Uptake of MDT

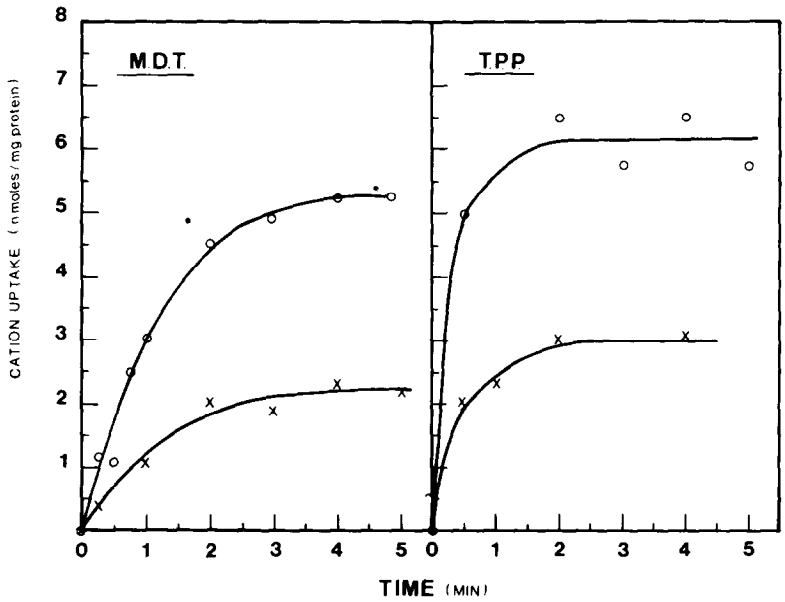

Fig.2. Time-course of MDT and TPP uptake. (○) In the presence of 10 mM ascorbate and 100 μM PMS. (X) No electrondonor added. The final concentrations of MDT and TPP were 100 μM.

occurred somewhat slower than that of TPP, but the final steady state was reached within 5 min. Steady state levels were reached, which were several fold higher than those observed under non-energized conditions. Assuming that like TPP, MDT passively equilibrates according to the $\Delta\psi$, the membrane potentials (inside negative) calculated from the difference in steady state levels of accumulation under energized and non-energized conditions are -68 ± 2.5 mV ($n = 7$) for MDT and -72 ± 2.5 mV ($n = 2$) for TPP. These values are in good agreement with the membrane potential observed [18] under identical conditions. The MDT concentration did not influence this value up to 150 μM. However at 200 μM a significant lowering of the membrane potential was found. No accumulation of the related compounds MT and NMA could be observed under non-energized nor under energized conditions (results not shown). It is of interest that rapid uptake of MT and MDT was found in animal cells [13,19], but the mechanism of transport remained unclear.

3.3. *Effect of ionophores*

The uptake of MDT and TPP in the presence of ascorbate-PMS was inhibited to the non-energized level in the presence of the potassium ionophore valinomycin (fig.3), which is known to diminish the $\Delta\psi$ by producing a potassium flux. A similar inhibition was observed in the presence of the uncoupler FCCP (fig.4), which acts as a proton ionophore and abolishes both ΔpH and $\Delta\psi$. On the other hand, nigericin, a proton–potassium antiporter, has hardly any effect on the ascorbate-PMS energized uptake of MDT (fig.4). In the presence of nigericin the ΔpH is dissipated by an electroneutral exchange of H^+ against K^+. A membrane potential of -70.5 mV could be calculated under these conditions.

3.4. *Uptake of MDT driven by a valinomycin–potassium diffusion potential*

When MDT is accumulated in response to a membrane potential (inside negative), it should be possible to drive MDT uptake by an artificially generated membrane potential. Such a membrane potential is induced by a valinomycin mediated potassium efflux. Figure 5 shows that indeed accumulation of MDT can be driven by such a diffusion potential and that the maximum level of uptake increases with increasing magnitude of this potential. Similar results have been obtained with TPMP [9]. The transient nature

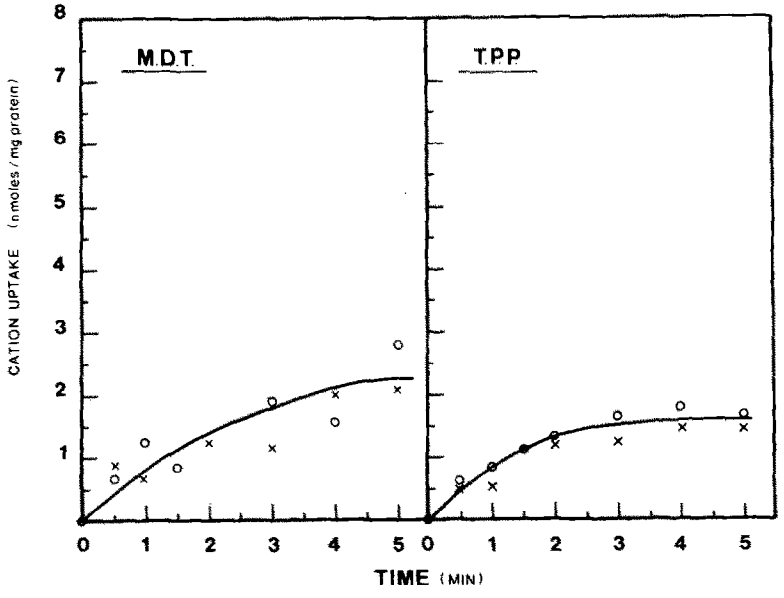

Fig.3. Effect of valinomycin on MDT and TPP uptake. (○) In the presence of 10 mM ascorbate and 100 μm PMS. (X) No electrondonor added. The final concentration of MDT was 100 μM; of TPP 40 μM. Valinomycin was added to a final concentration of 4 μM.

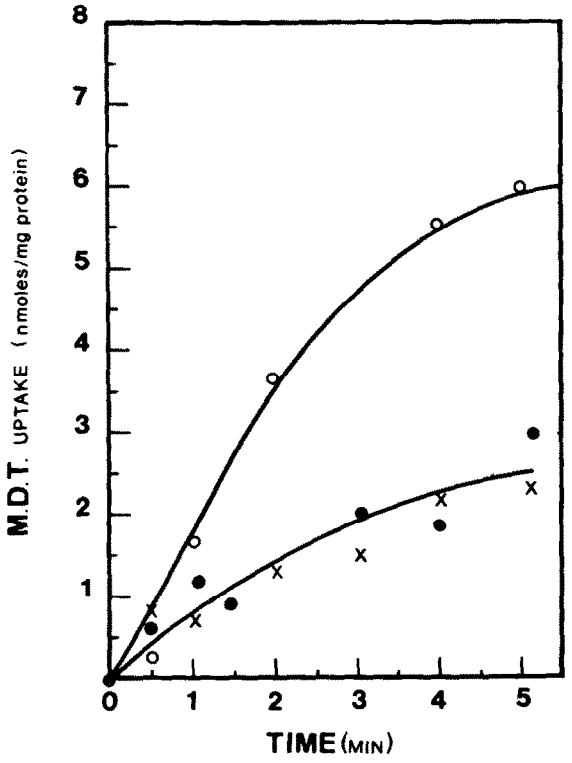

Fig.4. Effect of nigericin and FCCP on MDT uptake. (○) In the presence of 10 mM ascorbate, 100 μM PMS and 2 μM nigericin. (●) In the presence of 10 mM ascorbate, 100 μM PMS and 2 μM FCCP. (X) No electrondonor added. The final concentration of MDT was 100 μM.

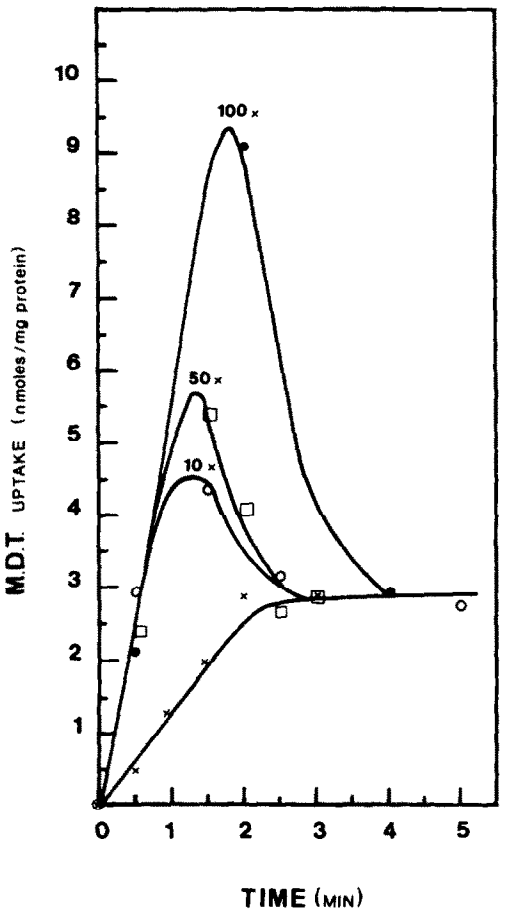

Fig.5. Uptake of MDT in response to a valinomycin–potassium diffusion potential. The vesicle suspension was diluted 10X (0), 50X (□) and 100X (●) (control: X). The final concentration of MDT was 100 μM.

of the uptake is most likely due to secondary ion movements.

4. Discussion and conclusions

The results demonstrate that MDT, just like TPP, equilibrates across the bacterial vesicle membrane in response to a $\Delta\psi$. MDT is accumulated to the same extent as TPP; accumulation is inhibited under conditions of decreased membrane potential (in the presence of valinomycin or FCCP) but not under conditions of decreased pH gradient (in the presence of nigericin). Moreover MDT is accumulated when a valinomycin–potassium diffusion potential is imposed across the vesicle membrane. The kinetics of uptake of MDT are in the same order as those of TPP. It is concluded that MDT distributes according to the membrane potential and that the drug is a useful probe for $\Delta\psi$ measurements. It has the advantage that it can be easily synthesized from commercially available precursors.

The low partition coefficient of MT and NMA in contrast to the high partition coefficient of MDT and the observation that MT and NMA are not accumulated by membrane vesicles of *Escherichia coli* ML 308–225, indicate that the sidegroup of the tropine part determines to a large extent the lipophilic properties of MDT.

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