# THE TRANSPORT OF D-LACTATE BY MEMBRANE VESICLES FROM *PARACOCCUS DENITRIFICANS*

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## 1. Introduction

The mechanism of respiratory-driven active transport in bacterial membrane vesicles has been the subject of much debate [1,2]. A number of recent reports [3–5] have presented compelling evidence for the view that, generally, in order to stimulate active transport in bacterial membrane vesicles, an externally-added physiological electron donor must first cross the vesicle membrane to gain access to its site of oxidation. The electron donor may then be oxidized by its specific dehydrogenase coupled to the respiratory chain; the available energy only then being capable of driving active transport.

Although it has been observed that D-lactate is an effective electron donor for driving the active transport of glycine in membrane vesicles from *Paracoccus denitrificans*, White et al. [6] in their preliminary survey, claimed that monocarboxylic acids were not transported by these membrane vesicles. The data presented in this communication however show that, in our hands, membrane vesicles derived from *P. denitrificans* do in fact catalyse the transport of D-lactate, although the time course of this uptake is very short. In addition, we show that the transport of D-lactate is sensitive to the thiol reagents *p*-chloromercuribenzoate (PCMB) and *N*-ethylmaleimide (NEM), indicating that the process is mediated by a specific transport system.

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## 2. Materials and methods

### 2.1. *Preparation of membrane vesicles*

*Paracoccus denitrificans* (NCIB 8944) (previously called *Micrococcus denitrificans*) was grown to 30°C on the complex medium of Scholes and Smith [7] with glutamate (50 mM) as carbon source in place of glucose. Aeration was provided by shaking in a New Brunswick Gyrotary Shaker G-25 at speed setting number 8 (250 rev/min, throw diameter of 2.4 cm). The cells were harvested and membrane vesicles prepared as described in [8], with the modifications adopted previously [9]. A further modification to this procedure was as follows: after the osmotic lysis of spheroplasts, membrane vesicles obtained were washed and finally resuspended in 10 mM Tris-acetate (pH 7.3) i.e. in the absence of $Mg^{2+}$ ions. Membrane vesicles (8.0 mg protein/ml) were frozen in liquid nitrogen and stored under liquid nitrogen for periods of up to two weeks. Before use, frozen vesicles were thawed at 30°C and subsequently maintained on ice for up to 4 h.

### 2.2. *Uptake studies*

The method was based on that described by Kaback [10]. Reactions were performed at 30°C in pre-warmed glass test tubes (7.5 cm × 9 mm internal diameter) sealed with a rubber bung. Reaction mixtures (final volume, 105 μl) were stirred by magnetic stirring bars (4 mm × 1 mm diameter). Components of the reaction mixture were added as the following: 50 μl, 20 mM Tris-phosphate (pH 7.3)/2 mM magnesium acetate; 20 μl membrane vesicle suspension (8.0 mg protein/ml); 10 μl, 0.2 M L-ascorbic

acid (adjusted to pH 7.0 with $NaHCO_3$); 10 μl water. The reaction mixture was then flushed with water-saturated oxygen for 2 min. Oxygen entered and left the test tube via two syringe needles which pierced the rubber bung. The oxygen inlet was directed vertically downwards from 0.5 cm above the surface of the reaction mixture. It was found that the high degree of oxygenation obtained in this manner was necessary for the demonstration of concentrative uptake in these vesicles powered by the electron donor system ascorbate plus *N,N,N',N'*-tetramethyl *p*-phenylenediamine (TMPD). The reaction was started by the addition of 10 μl, 1 mM TMPD and immediately afterwards, 5 μl [$^{14}$C] D-lactate (8 Ci/mol) were added to the final concentration 15 μM using a syringe fitted with an adjustable stop. The bung was then rapidly replaced. For the zero time sample, 10 μl water were added in place of TMPD and the reaction terminated immediately after the addition of [$^{14}$C] D-lactate.

Reactions were terminated at times indicated by rapid dilution with 2 ml, 0.1 M LiCl/1 mM $MgCl_2$ pre-equilibrated to 30°C, and immediate filtration (Gelman GA-8 filters, pore size 0.2 μm, diameter 25 mm). Filters were washed with 2 ml, 0.1 M LiCl/1 mM $MgCl_2$ at 30°C and dried for 1 h at 100°C. Dried filters were counted for radioactivity in 6 ml scintillation fluid (6 g 2,5 diphenyloxazole (PPO), 75 mg *p*-bis-(2-(5-phenyloxazolyl)-benzene, (POPOP) in 1 litre toluene) at an efficiency of 68% using a Packard Tricarb model 3003 liquid scintillation spectrometer.

### 2.3. *Miscellaneous*

Oxygen uptake rates were determined polarographically at 30°C [11]. PCMB was dissolved to 1 mM by heating in water at 65–75°C for 1 h and the pH adjusted to 7.0 with 1 M NaOH [12]. Protein was determined by the method of Lowry et al. [13], using bovine serum albumin as standard.

[U-$^{14}$C] D-lactic acid (sodium salt) was obtained from the Radiochemical Centre, Amersham, Bucks.

## 3. Results

Fig.1. shows that membrane vesicles from *P. denitrificans* rapidly accumulate D-lactate when oxidizing the electron-donating system, ascorbate

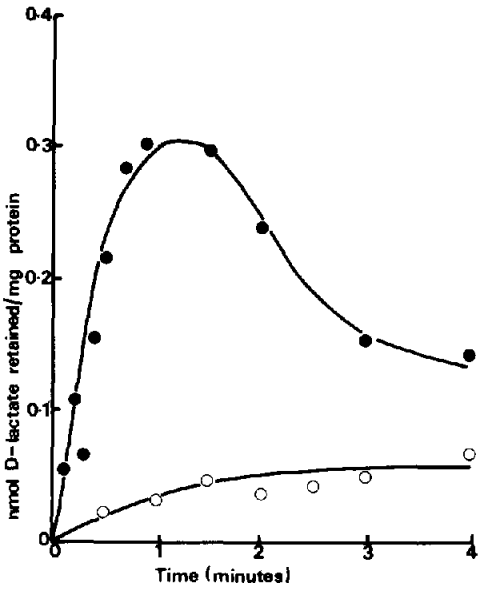


Fig.1. Time course of D-lactate uptake by membrane vesicles derived from *P. denitrificans*. (•) [$^{14}$C] D-lactate transport was assayed as described under Materials and methods. (○) Uptake of [$^{14}$C] D-lactate in the absence of electron donor: 10 μl water added in place of ascorbate and 10 μl water added in in place of TMPD.

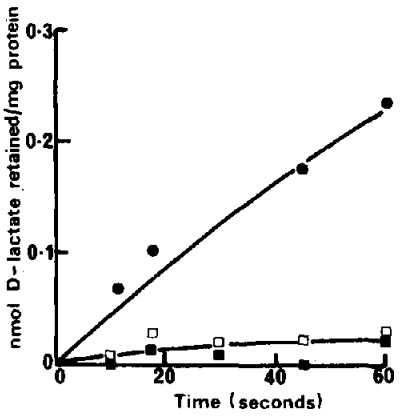


Fig.2. D-lactate uptake by membrane vesicles derived from *P. denitrificans*: the effect of thiol reagents. [$^{14}$C] D-lactate transport was assayed in a final volume of 105 μl as described under Materials and methods in the presence of the following: (•) 2 μl absolute ethanol added at t = −2 min. (□) PCMB added as 10 μl aqueous solution (final concentration 95 μM) at t = −2 min. (▪) NEM added as 2 μl ethanolic solution (final concentration 4.75 mM) at t = −2 min.

Table 1
The effect of thiol reagents on the oxidation of ascorbate plus TMPD by membrane vesicles derived from *P. denitrificans*

| Inhibitor | Oxygen uptake rate (ngatom oxygen/min/mg protein) | Comparitive rate (%) |
|---|---|---|
| Control (no additions) | 612 | 100 |
| + PCMB | 360 | 55 |
| + NEM | 558 | 90 |

Oxygen uptake rates were assayed at a protein concentration of 0.13 mg/ml in 10 mM Tris-phosphate (pH 7.3), 1 mM magnesium acetate. Ascorbate was added as 0.2 M solution (adjusted to pH 7.0 with sodium bicarbonate) to 20 mM final concentration. TMPD was added as 0.1 M solution to 100 μM final concentration. The above respiratory rates are corrected for non-enzymic auto-oxidation of ascorbate plus TMPD which was, in each case, 66 ngatom oxygen/min. PCMB (see Materials and methods) was added to 100 μM final concentration. NEM was added as 0.25 M ethanolic solution to 5 mM final concentration. Ethanol alone had no effect on the control rate of oxygen uptake.

plus TMPD in the presence of an adequate concentration of dissolved oxygen. Furthermore, the uptake of D-lactate is complete after approx. 1 min and immediately thereafter, the accumulated radioactivity effluxes.

The initial phase of this transport may be followed in order to determine the effects of a variety of inhibitors. Fig.2 shows that the thiol reagents NEM and PCMB both inhibit D-lactate transport to 13% of the control value (on an initial rate basis). Table 1 shows that the rate of oxidation of ascorbate plus TMPD by membrane vesicles is relatively unaffected by NEM at the concentration which strongly inhibits D-lactate transport. PCMB inhibits (to 50% of the control rate) oxygen uptake by membrane vesicles from *P. denitrificans* in the presence of ascorbate plus TMPD. However, this inhibition is a great deal less than that observed for D-lactate transport at the same concentration of PCMB.

## 4. Discussion

White et al. [6] have observed that D-lactate is an effective electron donor for driving active transport of glycine in membrane vesicles derived from *P. denitrificans* and we have confirmed this observation (unpublished). For the reasons described in the Introduction, we therefore decided to study the permeability of membrane vesicles from *P. denitrificans* to D-lactate.

As shown in fig.1, D-lactate is actively transported by membrane vesicles from *P. denitrificans* although the accumulated radioactivity effluxes rapidly after approx. 1 min. The cell membrane from *P. denitrificans* possesses an active respiratory-D-lactate dehydrogenase [14]. It is therefore probably that the transported D-lactate is rapidly converted to pyruvate within the membrane vesicles, as has been observed for membrane vesicles from *E. coli* [15]. We suggest therefore that the efflux of radioactivity observed in fig.1 represents efflux of pyruvate from the vesicles. Thus, it must be stressed that investigations of D-lactate transport in membrane vesicles from *P. denitrificans* must be carried out over short time periods.

It is unlikely that the observed initial rate of D-lactate uptake (0.38 nmol/min/mg protein) by membrane vesicles from *P. denitrificans* is due solely to passive transmembrane diffusion of D-lactic acid. Fig.2 and table 1 indicate that D-lactate transport is, in fact, mediated by a transport system which is sensitive to reagents which block free thiol groups. This observation agrees with the conclusions of Matin and Konings [15] that the active transport of D-lactate is mediated by a specific transport system in the respective membrane vesicles derived from several bacteria.

The data described in this report show that the transport properties of membrane vesicles from *P. denitrificans* do not conflict with the mechanistic proposals outlined in the Introduction; D-lactate is able to cross the membrane of vesicles derived from *P. denitrificans.* Furthermore, since the respiratory dehydrogenases of *P. denitrificans* are believed to be internally located in 'right-side-out' membrane vesicles [16], it appears that D-lactate transport is the necessary first step (preceding oxidation) in the mechanism of D-lactate-driven, glycine transport.

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